Tree Transgenesis

M. Fladung D. Ewald (Eds.)

Tree Transgenesis

Recent Developments

With 19 Figures, 8 in Color, and 23 Tables

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Preface

A continuous development in plant biotechnology including gene technology has been observable during the past 20 years. Different methods elaborated with model plants were also applied to forest trees on a larger scale. Whereas in the beginning the meaning of the term "plant biotechnology" embraced a wide variety of meanings like, e.g., regeneration of plantlets via tissue culture, embryo rescue, somatic embryogenesis and gene transfer, the focus of this term has changed more and more. Nowadays, it is the transfer of genes which comes into mind when plant biotechnology is discussed, including of course the evaluation of all challenges and risks related to gene transfer methods.

Compared with annual plants, especially in the field of agriculture, the work and the progress with transgenic trees is still in its infancy. Nevertheless, but often unnoticed by the scientific community, there are a few countries which already allow the commercial use of a restricted number of transgenic tree clones after different critical steps of approval. This and the ongoing improvement in transgenic research in trees led to the idea of preparing a summary of the present state of the art from different points of view. With the help of a number of authors directly or indirectly involved in tree transgenesis, this book was produced. Based on scientific results it is aim of this book to inform the reader about the present state of the art and to stimulate discussion concerning problems of biosafety and risk assessment and the necessary experimental tasks in the future, as well as to support decision-making processes in politics.

In view of the availability of the whole genomic sequence of poplar (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and also, in the near future, of *Eucalyptus* (http://www.ieugc.up.ac.za/ieugc_Main.htm#), gene technology is a valuable scientific tool to down-regulate or over-express single genes and, thus, study their role in plant growth and development. Such a functional genomics approach will allow us to unravel the basic principles of plant growth regulation one day. Thus it will soon be possible to improve transgenic trees mimicking natural strategies including their use for a sustainable application.

However, trees also need our special attention as unbred and long living individuals. In most cases, forests consist of wild populations of trees with great importance regarding both the climate and the sustainable provision of wood. Therefore, it is justified to take special care concerning risk assessment

and biosafety issues to prevent an undesired environmental release of transgenic trees by chance.

There is still an urgent need for ongoing research in the field of biotechnology in the near future. All aspects have to be included in these research strategies not only to estimate the risks properly but also to come to a critical evaluation concerning chances and challenges of transgenic trees to meet the future growing demand of the renewable resource wood.

April 2006

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Part A Transgenic Trees in the World

1 Field Trials with Transgenic Trees – State of the Art and Developments

MARCEL ROBISCHON

1.1 Introduction

Research and development on transgenic trees differs from such work carried out on herbaceous model systems first in that it necessarily involves field trials if data on aspects of the mature plant are required. Second, in contrast particularly to field tested transgenic agricultural crops, GM tree field trials are bound to be extended with the same plant individuals over longer than one single vegetative period and can last many years.

Given this, and taking into account the fact that in all cases a large amount of work has to be done before beginning any work beyond the test tube stage in the growth room and a potted plant in a greenhouse, the development of field trials and field releases worldwide is expected to be an indicator for overall development in the field of forest biotechnology.

1.2 Transgenic Trees in Test Tube and Field Trials

A field trial is expected to document in itself a well-developed research project that has led past various testing phases in lab based work to a stage in which the tree can be taken to the next round of tests in the field. It is, however, not just the success of the primary lab-based work that, under consideration of all the other factors, influences what happens in the field. The success of the field trials will also determine whether in the future more work is invested in the lab-based work. The dimensions of field releases of transgenic trees in trials can therefore only with great care be seen as a direct, simple function of the progress made in development in the lab. Many other factors come into the equation.

The closer research and development with transgenic trees gets to the field trial or release and thus the closer it gets to structures of primary production in "classical" forestry, the more it carries on some of the burdens of technical "peculiarities" and socio-economic involvements that are typical for forestry

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worldwide. On the technical and economics side, long production times can be identified that on the one hand cause a low return – whether in a classical management scenario or in the development of a new GM-tree based product – and on the other hand delay the progress of research and development considerably (Speidel 1984). On the social side the involvement of many stakeholders is typical and is found in either, e.g. the afforestation of a stand near a settlement or the start of a field trial (Köpf 2002).

This latter point is well reflected in the fact that a transgenic tree, if studied as a "tree" rather than a "seedling-like plantlet in a test tube" with the release of a transgenic plant in the long term and some potential environmental implications causes a great deal of concern to the public, as documented in a flood of non-technical and newspaper articles, media reports, political and lobbying activities and in some cases vehement protests (Arthur 1999; Highfield 1999; Miller 2003).

Keeping in mind the aforementioned limitations, the number and type of field trials, and the development of these data over time give an impression of both, work on GM trees already carried out successfully at an earlier stage of the development process, but also gives an impression on what further research may build up and, if in the context of present economic and political developments, into what directions future work may be pushed. In the following it is attempted to provide an overview of past and present field trials worldwide, with the aim of developing an image that allows some insight into the future developments that may shape work on transgenic trees.

The global situation regarding releases of transgenic trees to the field is nowhere documented completely and in detail. The main reason for this is a distinct lack of data and information. This is partly due to the nature of some data as "confidential business information" as in recent years a large percentage of field releases were carried out by the Research and Development labs of large forest companies, particularly outside Europe. Some companies, when approached by researchers or journalists, clearly stick to a "no-information" policy, leaving requests ignored and questions unanswered.

Obtaining information is in many cases a particular challenge, as the respective companies are often joint ventures between various other firms, often in the pulp and paper industry, or other industrial branches and are subject to frequent change by merger, takeover, sale, closure, re-naming etc. or partners leaving the joint venture. Under such circumstances it can also be difficult to trace back the historic continuity of work carried out by individual companies. This is true not only specifically for firms that carry out GM work on trees, but also for other companies in the field of industrial and plantation forestry (Carrere and Lohman 1996).

Some insight however is possible due to the legal and administrative structures in some countries that require permission for field trials and list releases together with some limited information in publicly accessible databases.

Therefore, for the following overview several sources that are quite different in nature have been used. For the US there is a detailed database listing all applications for permission, and respective notifications of a field release of a transgenic organism, which also includes trees (http://www.aphis.usda.gov/brs/status/BRS_public_data_file.xls). Equally detailed is the Canadian database published for all field releases of transgenic plants online at http://www.inspection.gc.ca/english/plaveg/bio/triesse.shtml. The same field releases are also partly covered in a database that lists the equivalent applications for Europe, US, Canada, Australia and New Zealand that is provided by OECD (Organisation for Economic Co-operation and Development) (http://www.olis.oecd.org/biotrack.nsf). The situation in the EU is separately documented in an EU database (http://biotech.jrc.it/deliberate/dbcountries.asp and http://gmoinfo.jrc.it/gmp_browse_geninf.asp).

While these databases are thought to be comprehensive, they do not give any specific information on the size of the respective field trial nor on whether this trial has in the end actually been carried out, or indeed at what point in time it has actually been terminated. They also do not show, whether an application for or a notification of a field trial is for a completely new experiment or simply the continuation of an earlier experiment with plants of the same type – or even the same plant specimens.

However, the regulative frameworks in many countries are at present still being developed. In these cases information was sought on work carried out in the respective country via academic networks. This data is backed up with information from scientific publications, non-technical publications and newspaper articles, environmentally concerned publications as well as personal communication with researchers and persons involved in environmental NGOs (non-governmental organization). It is an inherent problem of the evaluation of a range of diverse sources that the information obtained may in some cases not match or even be contradictory.

In this overview, first work on forest trees is covered. This includes species whose traditional use falls in either of the three classical functions of managed forests: production of timber and non-timber forest products, protection of the landscape, and the recreational function (Dieterich 1953). Trees whose main function is the production of fruit are discussed in a separate section. In addition there are also a few examples of genetically engineered trees in field trials, whose potential economic application is in the production of an entirely new product or service that is only tenuously linked to the traditional use of trees in forestry and fruit farming.

There are a few examples of transgenic trees that have been genetically modified to improve their use for ornamental, landscape or environmental purposes, which however do overlap with the function of creating a more productive forest crop. There is one field trial documented for *Amelanchier*, which has mainly ornamental use. In numbers such trials however are completely irrelevant and are mentioned here solely for completeness.

1.3 Transgenic Trees for Improvement of Forestry

1.3.1 Northern America

The region in which the largest number of field trials on transgenic trees has been carried out is North America.

Even though a country with traditionally strong research in forest biology, the share of field releases of transgenic trees in Canada is small. The Canadian database lists for 1997 a poplar with an antibiotic resistance released in Quebec (the only one found also in the OECD database), for 1998 a submission for herbicide tolerant poplar in Alberta, and from 2000–2004 two submissions for black spruce with selectable marker genes, an insect-resistant white spruce and a poplar with a selectable marker. These trials are well covered in the non-technical media. A "National Post" article of 2003, for example, covered a planned field trial with transgenic trees (Jack 2003). The trial comprises 400 transgenic spruces and poplars planted out in a forest near Val Cartier, Quebec. The article pointed out that there were as yet no commercial plantings of transgenic trees in Canada, but that the development by now had reached a point at which use in commercial plantations was within reach. This work was also publicised in CBC (Canadian Broadcasting Corporation) News (2003), quoting Armand Séguin of the Canadian Forest Service, according to whom this was the only field trial with transgenic trees in Canada.

The vast majority of field trials in North America to date took place in the US, for which the database (March 2005) documents about 185 applications respectively notification for releases of genetically engineered forest trees (Table 1.1).

Table 1.1. Present number of field trials with transgenic fruit and forest trees comparing Europe and North America

	Fruit trees Europe	Forest trees Europe	Fruit trees North America	Forest trees North America
Marker	–	7	3	45
Herbicide resistance	–	3	–	45
Insect resistance	–	1	13	15
Disease resistance	11	1	47	12
Sterility	–	1	3	28
Lignin	–	8	–	27
Developmental	6	4	3	27
Heavy metal	–	3	–	6
Fruit quality	–	–	21	–
Other	4	2	3	5
Total	21	30	94	212

For comparison in this paper these trials were grouped according to the nature of the altered trait (herbicide tolerance, insect resistance (Chap. 12), disease resistance (Chaps. 10 and 11), sterility or altered fertility (Chap. 2, Sect. 2.4), lignin content (Chap. 5), developmental traits, heavy metal tolerance (bioremediation, Chap. 7), or other traits. A clear change over time in the type of traits for which field trials were applied for respectively notified could be observed.

The work on *herbicide resistance*, for example, so far "peaked" in 1999 (Table 1.2) with the number of trials for this trait decreasing since. With the long term investments that forestry naturally involves (Speidel 1984), the altered trait has to be of potentially high economic significance. This may partly explain, for example, the reduction of experiments on transgenic trees with herbicide resistance. The then director of Weyerhaeuser forest biotechnology was quoted in a 2002 article in *Science* (Mann and Plummer 2002) with the comment that herbicide application in the forest industry "*is not that large of an expense*". Shifting to different herbicides if necessary may therefore, in the long run, be more economic than generating trees resistant to one particular to allow its extended use. Furthermore, the use of herbicides is a classical environmental issue and hence likely to form a focal point of public criticism.

Table 1.2. Applications and notifications of field trials using transgenic forest trees in the US. The category "other" includes work on gene stability and thus reflects also work on safety aspects. In most cases a release of a plant with a specific trait of interest is accompanied by a release of plants with markers or plants may have more than one trait, including the (visual) marker

Year	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
Type of trait																	
Marker	1	–	–	–	–	–	–	–	1	1	4	6	10	4	21	31	8
Herbicide resistance	–	–	–	–	–	1	1	1	2	7	13	7	3	7	2	2	1
Insect resistance	–	1	1	–	1	–	–	–	2	3	1	5	1	3	–	11	–
Disease resistance	–	–	–	–	–	–	–	–	2	3	2	1	–	–	1	4	–
Sterility	–	–	–	–	–	–	1	–	1	–	–	2	4	–	6	17	–
Lignin	–	–	–	–	–	–	–	–	–	2	–	2	–	–	6	10	4
Developmental	–	–	–	–	–	–	–	–	–	1	1	–	–	1	4	16	4
Heavy metal	–	–	–	–	–	–	–	–	–	–	–	–	1	–	2	3	–
Other	–	–	–	–	–	–	–	–	1	1	–	–	–	1	–	7	1
Total	1	1	1	–	1	1	2	1	9	18	21	23	19	16	42	10	18

Insect resistance has remained a trait worked on quite continuously with some of the earliest trials being on this trait, but still in 2004 a large number of field trials were carried out with trees modified for insect resistance. This can be easily explained with pest damage being a continuous problem in forestry, in particular given the steady stream of exotic species being introduced into new environments as novel pests (Schedl 1936) and the enormous cost arising from this ongoing "biological globalisation" (Scigliano 1999). In an attempt to make an informed guess of future development work on this trait, it has to be taken into account that a large proportion of the earlier work on insect resistance in trees was carried out with the Bt (*Bacillus thuringiensis* toxin) genes. However, in case it turns out that extended use of Bt-transgenic plants leads to the formation of resistance in pests, for which indication has already been found (ABC News 19th April 2001), the concept of achieving insect resistance may have to be revised. This may lead to more research and more field trials being required in the future.

Work on *disease resistance* seems to follow a similar development, without however the very earliest trials in the early 1990s. After the 1990s the number of trials with this trait seemed to decline. However, even in 2003 and 2004 some trials for this trait appeared again. This is partly due to transgenic methods now under discussion with the aim of healing the wounds that disastrous epidemics have torn into stands of chestnuts in the forests of North American East Coast, or to bring back elms to the suburban streets after they was all but wiped out from natural and cultural landscapes in America and Europe (Campanella 2003). So far there is one field trial with transgenic elm and two field trials with transgenic American chestnut documented in the US database. It is likely that work on this will continue, given the high importance these tree species once had in the Eastern North American landscape (Dr. R.C. Kellison, personal communication[1]). Furthermore, there is also work on transgenic lines of the Chinese Elm (Aziz et al. 2003) suggesting a substantial interest in this problem.

Notably, work to generate transgenic, disease resistant elm has also been carried out in Europe (Gartland et al. 2000) and has attracted considerable attention from the media (Kelbie 2001), as yet however without any field trials. More details on this topic are given in a chapter later in this volume. Work on transgenic lines has also been published for European Chestnut (da Costa Seabra and Pais 1999). It has, however, as yet not lead to field trials, possibly because the problem of chestnut decline does not have the same dimensions as in the US (Prof. Dr. O. Holdenrieder, personal communication[2] and Dr. U. Heiniger, personal communication[3]).

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An increased interest in research in trees with altered *lignin composition* may be interpreted in two ways. First, there is a rise in the production of pulp and paper worldwide. A FAO (United Nations Food and Agriculture Organisation) forecast predicted a growth of global paper consumption to 400 million tonnes by 2010. For comparison, the consumption in 1970 was estimated at 125 million tonnes and 1995 at 276 million tonnes (Enskilda Futures 1997).

Second, the production of paper is a procedure with extremely severe environmental effects. Even though technical progress, including a number of biotechnical developments (Bajpai and Bajpai 1998), aims at improving the situation, it is expected that with the rising paper productivity worldwide these problems will increase, making the development of trees with lower lignin content an economically and environmentally interesting topic (Dr. K. Holt, personal communication[4]).

The importance of work linked to *bioremediation* is obvious with large areas of land being polluted by industrial waste products, including heavy metals (Raskin and Ensley 2000). Since 2001, field trials have been notified for work on transgenic trees with the ability to tolerate heavy metal contamination of the soil. From 2001 to 2004 their number increased from 1 to 3.

The work on *sterility or altered fertility* of trees has increased clearly over recent years. There are two possible reasons for this. First, still in the context of increased demand for wood products, particularly pulp and paper, a reduced fertility is expected to increase the productivity of the tree. Second, environmental reasons are likely to play an increasing role for research in this trait. It is in the interest of both, publicly funded institutions as well as private companies to work on methods to reduce unwanted gene flow from a transgenic crop into natural populations or to prevent uncontrolled spreading of transgenic material.

The somewhat widely defined category "developmental traits" includes work with the aim of increasing yield, but also work that is involved in basic research, onto which more applied projects may build (e.g. nitrogen metabolism (Chap. 8) or disease resistance traits (Chaps. 10 and 11)). Finally, field trials established to study biosafety-related issues like gene or genome stability (Chap. 14) or horizontal gene transfer (Chap. 15) fall in this category. Such research is important in the frame of elevating public acceptance.

It can be concluded that it is evident from the sources used that the number of field trials in North America, as well as the number of traits worked on, has been growing since the first trials at the beginning of the 1990s. There has also been a clear shift in the importance of individual types of traits. This shift can be linked well to the economic and political context. Overall in the work documented in the database for the US, a trend towards the development of a more "sophisticated" and more elaborate use of molecular biological

[4] Dr. Karen Holt, Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK

methods for potential use in plantation forestry, that also takes into account environmental concerns, can be observed.

1.3.2 Europe

In the EU, according to the EU and OECD databases, there have been about 30 applications for field trials with transgenic forest trees to date. This included 18 on poplar, 4 on eucalyptus, 2 on pine and 2 on spruce. The distribution according to types of traits is given in Table 1.3.

Insect resistance, herbicide tolerance and disease resistance are much less an issue in research in Europe than in the US. Work in field trials with trees modified with these aims appears to phase out around the same time as work with herbicide resistant trees. This can be interpreted in the context of a completely different structure of the forest industry in Europe and the US, with central aspects being the absence of large (i.e. "American dimension") forest companies in Europe (with the exception of Scandinavia). Also large areas of fast growing monoculture plantations to be clear cut after the rotation time for production of pulp and paper are more of an exception in wood production in Europe, with the exception of some fast growing eucalyptus plantations in Spain and Portugal.

Nevertheless, studies on lignin formation that were linked in the previous section – in an US-American scenario – to plantation forestry for pulp and paper, increased in numbers also in Europe over the years. Notably a 1997

Table 1.3. Applications and notifications of field trials using transgenic forest trees in the EU

Type of trait	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04
Marker	–	1	1	–	2	2	–	–	–	–	–	–	–
Herbicide resistance	–	1	–	1	1	–	–	–	1	–	–	–	–
Insect resistance	–	–	–	1	–	–	–	–	1	–	–	–	–
Disease resistance	–	–	–	–	–	–	–	–	1	–	–	–	–
Sterility	–	–	–	2	–	–	–	–	–	–	–	–	–
Lignin	–	–	–	2	2	1	–	1	–	–	–	1	–
Developmental	–	–	–	–	1	–	2	1	1	–	–	1	1
Heavy metal	–	–	–	–	–	–	–	–	–	1	–	–	–
Other	–	–	–	–	–	–	–	–	–	–	–	2	–
Total	–	2	1	6	6	3	2	2	4	1	–	4	1

In contrast to Table 1.2, in this Table marker genes are not listed as a separate trait, unless it is the only trait worked on in the specific experiment

trial on lignin was carried out by a large company involved in this type of forestry in Portugal (cf. Table 1.4). In other cases this interest in lignin formation may be due on the one hand to basic research with interest in the basic processes of lignin formation. On the other hand a driving force in Europe may be the interest to contribute in the long run to biotechnical mechanisms to reduce pollution caused by the paper industry (Dr. K. Holt, personal communication).

As in the US the work on developmental traits has set in relatively late but appears to be continuously an important topic worked on, potentially as a basis for future, more applied research.

With reference to work linked to bioremediation (Chap. 7), a trial carried out on transgenic poplar with altered glutathione level in Germany is of particular interest. These trees are supposed to help mopping up heavy metals from the soil (Dr. A. Peuke, personal communication[5]) and reflect a growing interest in environmental applications of transgenic trees in Europe.

The most obvious difference between the development in Europe and the US is that there is a much smaller number of trials and also no apparent trend

Table 1.4. Field trials on transgenic trees applied for or notified by industrial companies in the US and Europe

	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
USA total	–	–	–	1	1	6	9	11	15	11	33	65	10
Arborgen	–	–	–	–	–	–	–	–	3	10	30	60	9
Applied Phytogenetic	–	–	–	–	–	–	–	–	–	–	2	4	1
Westvaco	–	–	–	–	–	2	9	10	9	1	1	1	–
Int. Paper	–	–	–	–	–	–	–	1	3	–	–	–	–
Monsanto	–	–	–	–	–	1	–	–	–	–	–	–	–
Weyerhaeuser	–	–	–	–	1	–	–	–	–	–	–	–	–
Union Camp	–	–	–	1	–	3	–	–	–	–	–	–	–
Europe total	1	1	1	1	1	–	–	–	–	–	–	–	–

For the US a specification of the companies involved is given
In Europe the 1993 and 1995 trials have been carried out by Shell Forestry, now dissolved
The 1994 trials was by *Celulosas de Asturias* in collaboration with Advanced Technologies Cambridge
The 1996 trials was by Zeneca
The 1997 trial was by Stora Celbi – i.e. the five industrial trials in Europe carried out by four companies
The 152 industrial trials in the US were carried out by basically 7 companies
In recent years there is also an increasing centralization with the vast majority of field trials being carried out by one single company
Notably ArborGen is a joint venture of Westwaco, Fletcher Challenge, International Paper and Monsanto founded in 1999

[5] Dr. Andreas Peuke, Institut für Forstbotanik und Baumphysiologie, Universität Freiburg, Am Flughafen 17, D-79085 Freiburg

of an increase in their number. The development in Europe mirrors the trends observed in the US in as much as there has been over the years generally a growing number of different traits that have been worked on in transgenic trees. As in the US in recent years, work with traits that may be important for environmental purposes, namely resistance to heavy metals, have emerged.

As a consequence of the different structure of forest industry, a potentially different attitude in the public and the different legal environment, it can be expected that in the nearer future more companies will choose to do work in the US rather than in Europe. In this context it is of interest to compare the trials carried out to date by industry in Europe and in North America.

Of the trials documented in the databases for North America, 162 were, as far as is evident from the applications as listed in the databases, run by industrial companies. All of these were based in the US. The 152 industrial trials in the US were carried out by no more than 7 companies. In recent years there is also an increasing centralization with the vast majority of field trials being carried out by a single company (Table 1.4). Notably, ArborGen is a joint venture of Westwaco, Fletcher Challenge, International Paper and Monsanto. After its foundation in 1999 the applications and notifications of the mother companies phase out.

Of the 30 field trials with forest trees in Europe, as far as it is evident from the applications documented in the databases, only 5 trials were run by industrial companies (Table 1.4). All of these were early trials (between 1993 and 1997) on eucalyptus apart from one case with poplar. Of these industrial trials two carried out in 1993 and 1995 were run by Shell Forestry, now dissolved. The 1994 trial was by Celulosas de Asturias (CEASA) in collaboration with Advanced Technologies Cambridge. The 1996 trial was by Zeneca in a project that was more of an academic nature and run jointly with the French national institute for agronomy research INRA (Institut National de la Recherche Agronomique). The 1997 trial was carried out by Stora Celbi, i.e. the five industrial trials in Europe were carried out by a mere four companies. All the field trials in Europe were relatively short lived. The two trials with eucalyptus in England lasted for three months each. Also the trial at CEASA lasted for three to four months only. The trial by Astra Zeneca and INRA however was worked on for four years and was destroyed by activists shortly before the planned date of termination (Dr. C. Halpin, personal communication[6]). Future work on transgenic trees is not part of the business portfolio of Zeneca at present (Dr. K. Holt, personal communication). On the Stora-Celbi trial there is no further information available. Generally the interest in industry to conduct field trials in Europe appears to have faded away in the past. All field trials on transgenic forest trees carried out at present in Europe are part of academic studies.

[6] Dr. Claire Halpin, Plant Research Unit, School of Life Sciences, University of Dundee at SCRI, Invergowrie, Dundee DD2 5DA, UK

This development is illustrated in the history of Shell Forestry. According to Dr. J. Purse (personal communication[7]), it became clear in the late 1990s, that developing a GM tree crop was too expensive and not cost effective for one single company. Therefore the company initially tried to get involved into joint ventures – as for example with Sappi (South African Pulp and Paper Industries Ltd.) in South Africa (see below). The plan to transform the research branch, after the company's decision to withdraw from work on GM trees in order to obtain FSC (Forest Stewardship Council) certification and to provide the development of GM trees for other companies, failed due to lack of customers. The company withdrew not only from field trials but then from molecular work on trees and was eventually dissolved by the Shell concern altogether (Dr. J. Purse, personal communication).

1.3.3 Latin America

In the past there have been field trials of transgenic eucalyptus in Latin America, namely in Uruguay and Chile. These are however not documented in any of the databases mentioned above, nor is there any scientific publication on any of them. They are however covered in publications produced by environmental groups. The "World Rainforest Movements" (WRM) bulletin comments on field trials with transgenic trees (no species is mentioned, but from other sources it is documented as eucalyptus) run by 'Florestal Oriental', a forest company that then was then jointly owned by Shell and UPM (United Paper Mills Ltd.)/Kymene in Uruguay. According to Pérez (2000) these trials were run only over a period of two years and ended in 1999. All trees were according to this source destroyed. This information was confirmed by Dr. J. Purse, formerly of Shell Forestry. The company was at that time aiming at FSC certification, which would have been precluded if these experiments had been carried on longer. In 2000 there were no transgenic trees in the country (Pérez 2000).

Field trials with transgenic trees were also carried out in 1999 in Chile by a company called 'Forestal y Agricola Monte Aguila S.A.' that then belonged to the same group and was thus basically in-house research of Shell Forestry (Dr. J. Purse, personal communication). It involved a mere 60 eucalyptus plants that were resistant against the herbicide glyphosate. Notably these field trials in Latin America have attracted interest worldwide and references to these are found frequently in NGO literature concerned with environmental issues (Manzur 2000).

The traits of herbicide resistance and altered lignin formation relate as in the case of similar work in other parts of the world to plantation forestry with short rotation times for pulp and paper production. The small scale, short life and the fact that they were terminated without being continued or repeated

[7] Dr. John Purse, Prima Bio, Kent, UK

in this region, however, suggests that these trials were merely an initial test to try out for the first time the new technology by an individual company, but not part of a longer-term development.

In January 2001, Shell produced a press release (Royal Dutch Shell Petroleum 2001) stating that it had received FSC certification for its forest business in Latin America. FSC certification excludes not only the use of GM trees by a forest company in its plantations, but also involvement of the respective company in research. Shell has since withdrawn from this work and from the forest industry entirely.

It may be expected that, for the successor companies, the FSC certification is of importance, in particular with respect to the European market. With the dissolving of Shell forestry and their research branch, the direct link to the technical side of the development has vanished. It therefore does not seem likely that these or similar trials are going to be revived soon.

However, Chile actively supports the development of the biotech sector. This suggests that in the future this country may attract other investors for GM work on trees or may become involved in research with its own institutions.

1.3.4 South Africa

In the past at least one field trial with transgenic trees has existed in South Africa. This experiment was carried out with roundup-ready resistant eucalyptus planted in 1997 in a project run jointly by Sappi, Shell and Monsanto (Dr. J. Purse, personal communication). Dr. Arlene Bayley of Sappi[8] (personal communication) stated that this field trial was terminated after a year, due to a temporary limited permission. The plants herbicide tolerance was tested at an age of 6 and 10 months. According to Dr. Bayley (personal communication) Sappi was, however, at the time of these works, more interested in testing the technology and collecting experience with the legal and administrative processes than in a commercial use.

The situation with regards to transgenic field trials in South Africa is, however, not as easy to assess as in the case of the US and Europe. While a 2002 newspaper article from South Africa (Friedman 2002) quotes NGO Biowatch with the statement that, in 2001, permission was given for field trials on (among other crops) eucalyptus and apples[9], Mrs. M. Vosges[10], South African

[8] Dr. Arlene Bayley, Sappi Forest Research, PO Box 472, Kwambonambi 3915, South Africa

[9] Notably the above quoted article reports an NGO going to court to enforce a clearer information policy on releases of genetically engineered organisms in South Africa, which in itself reflects a lack of sufficient information and confirms the above described somewhat unclear situation. No comments however were received from Biowatch South Africa

[10] Mrs. Michelle Vosges, Directorate Genetic Resources, Department of Agriculture, Harvest House Room 261, 30 Hamilton Street, Arcadia, Pretoria, 0002, South Africa

Department of Agriculture as well as Prof. M. J. Wingfield[11], Director of the Forestry and Agricultural Biotechnology Institute of the University of Pretoria confirmed in personal communications (2005 and 2004, respectively) that there were to their best knowledge at present no field trials with transgenic forest trees in South Africa. This is supported by the fact that all forest companies in South Africa are FSC certified (Prof. M. J. Wingfield, personal communication[8], Dr. S. Verryn, personal communication[12]) which prevents not only commercial use but also research and development work with genetically engineered organisms.

For other parts of Africa there is no information on any cases of transgenic trees being released into the field. For the illegal release of material in Kenya, for example, that was recently reported in some newspapers and online magazines (N.N. 2004a,b) no convincing evidence has been produced as yet. According to the Director of ISAAA (International Service for the Acquisition of Agri-biotech Applications) AfriCenter, Dr. S. Wakhusama, personal communication[13] (2004), there are at present no genetically engineered trees in the country.

1.3.5 Australasia

1.3.5.1 New Zealand

Field trials on transgenic trees have also been run in the past in New Zealand. The OECD database lists two field trials on transgenic pine, probably radiate pine, applied for in 1997 and 2000. The first of these was carried out by the New Zealand Forest Research Institute, the second by the private company Carter Holt Harvey Pulp and Paper.

Work on transgenic *Pinus radiata* has a long tradition in New Zealand. The group of Dr. Christian Walter developed transformation methods for *Pinus radiata* over the last decade. The first transgenic plants were planted in a field trial in 1998. This trial was ended in 2003 due to expiry of the permission that covered only five years. At present, however, there are two further field trials with transgenic radiata pine going on in New Zealand, which are supposed to run from 2003 to 2023. The trees planted in this trial carry marker genes, herbicide resistance genes and genes linked to reproductive development. The research work is aimed at understanding environmental effects and there are no plans for commercialisation (Dr. C. Walter[14], personal communication).

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[11] Prof. Dr. Michael J. Wingfield, Mondi Professor of Forest Pathology, Director, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

[12] Dr. Steve Verryn, Council for Scientific and Industrial Research (CSIR), Meiring Naude Road, Brummeria, Pretoria, South Africa

[13] Dr. Sam Wakhusama, ISAA, Africenter, PO Box 25171, Nairobi 00603, Kenya

[14] Dr. Christian Walter, ForestResearch, Private Bag 3020, Rotorua, New Zealand

A driving force in research and development in New Zealand is the private industry, such as, for example, Fletcher Challenge. Some indication for further work going on at present, such as the efforts to sequence *P. radiata* cDNA in which private companies are involved (Dr. C. Walter, personal communication), suggests that there is an interest in industry in molecular work, and possibly also in taking it further to create transgenic trees. Generally in New Zealand the public opinion is critical towards GM field trials (Ms. J. Fitzsimons, personal communication[15]). The fact that Fletcher Challenge is involved in the joint venture ArborGen may suggest that some work in its interest is carried out in the US rather than in environmentally highly aware New Zealand.

1.3.5.2 Japan

In March 2004 Nippon Paper Industries Co., Ltd. announced the decision to test transgenic Eucalyptus trees with an increased stress resistance in a field trial in Japan (N.N. 2004c). According to the quoted article this was to be the first field release of a transgenic tree in Japan (N.N. 2004d).

This information was confirmed in a personal communication by Mr. P. Mouquet[16] of the French Embassy in Japan. At this point, however, there was as yet no information available as to whether this field trial was permitted by the Japanese authorities. According to Mr. Mouquet, a mission of French forest scientists to Japan was planned for October 2004 who also wanted to visit this experimental plot at Nippon Paper. A two page statement from the French Embassy's Service pour la Science et la Technologie produced later in 2004 by Mr. S. Roy (personal communication)[17] mentions an interest of industry in forest biotechnology, including genetic transformation, but does not provide any more information on this field trial. Further requests to Nippon paper were ignored.

Generally the policy in Japan is cautious in matters concerning transgenic material (Mrs. C. Haa, personal communication[18]), which suggests a slow development in this work, comparable to Europe. However, as yet there are too few cases and too little information to draw any valid conclusions.

1.3.5.3 Vietnam

The above-mentioned Nikkei Magazine also produced an article stating the existence of field trials with transgenic Eucalyptus in Vietnam. The existence

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[15] Ms. Jeanette Fitzsimons. Co-leader Green Party New Zealand, Freepost Parliament, PO Box 18888, Wellington, New Zealand

[16] Mr. Pascal Mouquet, Chargé de Mission Agronomie – Agroalimentaire, Ambassade de France au Japon, Tokyo

[17] Mr. Stéphane Roy, Attaché Scientifique, Ambassade de France au Japon, Tokyo

[18] Mrs. Cordula Haß, Botschaft von Japan, Hiroshimastr. 6,10785 Berlin

of field trials on *Eucalyptus camaldulensis* in this country was also pointed out by Prof. Dr. Rod Griffin[19] (personal communication).

Some information on these trials was shared in the course of this study by Dr. T. Hibino, Oji[20], who stated that the Oji Paper Forestry Research Institute executed the field test of a genetically modified eucalyptus with altered lignin biosynthesis and putatively altered pulping properties between March 1998 and December 2001 in cooperation with the Research Centre for Forest Tree Improvement (RCFTI), Forest Science Institute of Vietnam (FSIV) on their grounds. The field test was conducted to evaluate pulping properties. Some data were to be published via FSIV. From this institution, however, no further information was received, neither was information available as to whether there is any future work planned with GM trees. Generally for Vietnam – as well as for most East- and Southeast Asian countries – there is too little information available to develop an understanding of the potential for future development in work with transgenic tree field trials.

1.3.5.4 China

At the largest scale, releases of transgenic forest trees have been performed in the Peoples Republic of China (Chap. 2). A number of scientific publications document early work on poplar transformation (Wang et al. 1990). By today work on transgenic poplar in this country is so advanced that transgenic trees are not only released in field trials but are planted as a commercial crop in plantation forestry. Progress in research in this field in China is rapid and an increased number of trials and releases for commercial plantations can be expected, in particular in view of the country's ambitious afforestation projects (BBC News, March 3rd 2001; Richardson 1990) and the large areas of soil with limited fertility due to high salinity (Dr. J. Hu, personal communication[21] and Prof. Dr. M. Yang, personal communication[22]).

The topic of releases of transgenic trees in China is covered in detail in the chapter by Ewald et al. (Chap. 2) in this volume and is hence not further discussed here.

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[19] Prof. Dr. Rod Griffin, CRC for Sustainable Production Forestry, University of Tasmania, Private bag 12, Hobart, Tasmania, Australia 7001

[20] Dr. Takashi Hibino, Forestry Research Institute, Oji Paper Co. Ltd., 24–9 Nobono, Kameyama, Mie, Japan, 519–02

[21] Dr. JianJun Hu, Chinese Academy of Forestry, Wanshou Shan, Beijing 100091, Peoples Republic of China

[22] Prof. Dr. Minsheng Yang, Agricultural University Hebei, Baoding, No.1 Hezuo Road, Baoding City, Provinz Hebei 071002, Peoples Republic of China

1.4 Fruit Trees

1.4.1 North America

A considerable number of applications and notifications for field trials for fruit trees is documented in the above-mentioned databases for the US and Europe as well as in two cases for Australia and Canada respectively. As in the case of forest trees, the largest number of field trials with fruit trees has been applied for in the US (see Table 1.1).

The great majority of GM fruit tree trials in the US were carried out with apple and papaya with 35 and 21 trials, respectively. The work on papaya is a very special and interesting case that is covered later (Chaps. 3 and 9). There are also field trials with transgenic citrus trees in the US, with six trials on grapefruit alone, in which resistance both against viruses and against the insects that acts as a transmitting agent has been combined.

In addition, some fruits consumed generally in smaller quantities, such as avocado and lime, have been the object of work (Chap. 3). In 1998 Tao and Dandekar reported that a field trial with Bt expressing persimmon trees was in preparation. Indeed, eight notifications for field trials with this species are found for this year. The situation for tropical trees and tree-like species is described in detail in Chap. 4.

While there is relatively little documentation of trials on transgenic fruit tree in the USA in scientific publications or non-technical publications, the importance of field trials with transgenic apple was confirmed in a personal communication by Dr. H. Aldwinckle[23] at Cornell University, the institution by which most trials were notified. As these fruits, however, did not show the desired resistance against the pathogens, they have as yet not been developed for the market.

In 1996 and 1998 there were submissions for field trials with cherry in British Columbia, Canada. The 1998 submission is for altered fruit quality. Since then there have been no further trials with fruit trees documented in Canada.

1.4.2 Europe

The EU database also lists a number of applications for field trials on fruit trees. These, however, are much fewer in numbers. Of the field trials with fruit trees applied for in Europe, eight were to study apple, four pear, three cherry, two olive, two citrus and one plum.

An important trait is, as in the US, disease resistance, particularly virus resistance (Chap. 9). For field trials on sharka resistant plum that were

[23] Prof. Dr. Herb S. Aldwinckle, Cornell University, Department of Plant Pathology, Geneva NY 14456, USA

applied for in Spain, some documentation is found in the scientific literature. Ravelonandro et al. (2000) generated transgenic plum plants carrying a plum pox virus coat protein (PPV CP) gene causing resistance to the sharka virus. The authors state that these trees "*are being tested under natural conditions of infection in Skierniewice (Poland), Bistrita (Romania) and Valencia (Spain)*". In the same reference, two field trials are mentioned, which otherwise cannot be found documented anywhere. There is also work published on transgenic, virus resistant *Prunus armeniaca* in Austria, which, according to Dr. K. Pascher (personal communication)[24] was previously only tested in the greenhouse. No field trials are planned in Austria either.

In the case of an application for field trials with transgenic apple applied for by the Bundesanstalt für Züchtungsforschung an Kulturpflanzen, the refusal of permission was extensively covered in the press (Schuh 2003). There are at present no field trials with transgenic fruit trees in Germany; however, there is work planned in the Netherlands (Prof. Dr. V. Hanke, personal communication[25]) for this year.

Transgenic lines of apple and pear have been tested in the field in Russia (Dr. S. Dolgov, personal communication[26]) on which however no documentation is found in any databases. The trials are, however, mentioned in one non-technical publication by an American NGO (Schwartz 2000). More detailed information on work carried out on transgenic trees in Russia is given in the chapter by S. Dolgov and V. Hanke in this volume (Chap. 16).

Outside Europe and North America there have been only a small number of field trials on fruit trees. For 1994 there was a trial with apple carrying a marker gene recorded in Australia in the OECD database. Several trials worldwide have been run with papaya, which are covered later.

The field trials documented in the databases mirror clear differences between development in the US and Europe. In the US the industrial research, e.g., on apples is considerable, but this does not appear to play a role in Europe.

In Europe pressure against such trials is strong. The controversial issue of GM field trials is, in the public opinion, acerbated by the link to a potential GM food crop. This aspect in particular makes the situation an entirely different one from that in North America, given that in the US and Canada, apart from agricultural GM crops such as Bt maize and oilseed rape, only one GM tree fruit is on the market – a ringspot virus resistant transgenic papaya.

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[25] Prof. Dr. Viola Hanke, Bundesanstalt für Züchtungsforschung, Pillnitzer Platz 39, D-01326 Dresden

[26] Dr. Sergey Dolgov, Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Region Moscow, Russia, 142290

1.4.3 The Papaya Story

The development of transgenic papaya with resistance against the papaya ringspot virus is well documented in databases, scientific literature and non-technical publications (for details see Chap. 9). The development of the transgenic lines for other parts of the US territory, with different strains of the virus, is reflected, e.g., in an application of material transfer of transgenic papaya plants to the University of the American Virgin Islands and Puerto Rico. There is evidence for some field trials being carried out in Thailand, as reflected in a few environmentally oriented news articles, but also by publications from researchers working on this crop. Gonsalves et al. (2004) state that "*Starting in 1992, the technology transfer program has been implemented with agencies in the countries of Brazil, Jamaica, Venezuela, Thailand, and recently with Bangladesh and the east African countries of Tanzania, Uganda, and Kenya*." Field trials of different size have been started in Brazil, Jamaica and Thailand. A trial was also started in Venezuela, but destroyed by activists prior to data collection. While with papaya the field trials seem to continue in various parts of the tropics, commercialisation has not been achieved anywhere outside the US. According to Mr. M. Hall, USAID (United States Agency for International Development)[27] there are, for example, as yet no transgenic papaya plants in the field in Eastern or Southern Africa, even though there has been early work to develop a papaya specifically resistant to the ringspot-virus variant of the Lake Victoria region. The OECD database furthermore records two field trials in 1998 and 1999 with virus resistant papaya lines in Australia.

1.4.4 New Applications of Transgenic Trees

Within work to develop new applications of transgenic trees that are neither part of the classical applications of trees in forestry nor fruit farming, the development of transgenic rubber trees or banana as tree-like plant as a source for proteins is at present advancing (Chap. 3).

There have been small scale field trials in Malaysia (Dr. H.Y. Yeang, personal communication[28]), but as yet there are no concrete plans for commercialisation in this county in the near future. There are no databases documenting this work, nor has it been published as yet in international scientific journals or covered by non-technical press articles.

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[27] Mr. Mike Hall, REDSO/ESA, USAID/Nairobi, Unit 64102, APO AE 09831–4102, Kenya

[28] Dr. Hoong Yeet Yeang, Rubber Research Institute of Malaysia (RRIM), PO Box 10150 Kuala Lumpur, Malaysia

1.5 Conclusions

Transgenic trees have been tested in countries with such diverse structures in the forest industry as Canada or Finland, characterized by boreal climate and long rotation times, and South Africa with short rotation times and plantations for pulp and paper production.

However, in all cases there is a more or less direct link between the development of transgenic trees and plantation forestry. By far the most field trials with transgenic trees have been carried out with species that were modified in traits that were hoped to improve their value in "classic" uses of trees in plantation forestry. This includes resistance against common pests and diseases, herbicide resistance or reduced lignin content in order to improve the quality for the raw material of pulp and paper.

Particularly in the US, private companies are a major driving force behind a large body of work on field trials with transgenic trees. An example is ArborGen, founded as a Joint Venture of a group of companies including some of the largest global players in the forest industry such as *International Paper*, *Fletcher Challenge Forests* and *Westvaco Corp*. Outside the US there are at present only very few trials run by private companies, with one recent example in Japan, and just a handful of past trials in Europe, South Africa, Latin America and Australasia.

Many companies in the forest industry who in the past showed a keen interest in such technologies have now given up such work, such as Sappi, and it has not, as yet, apparently caused major economic harm to them. This indicates that in the present situation the forest industry can still do very well without GM tree crops. Furthermore, also within the US, the interest of private industry seems to be reduced (Mann and Plummer 2002).

Nevertheless, even in countries, in which there are at present only limited or no field trials at all, some interest in future work is evident and is demonstrated by continued work in lab-based research, including such costly and long-term projects as sequencing tree genomes.

With the 'middle step' of field trials appearing like a bottleneck, the 'final step' in the development from the lab bench to the market, the commercialisation, appears unlikely in most countries in the nearer future. The exception is the rather special case of China, where commercialisation of transgenic forest trees has already taken place.

The situation is slightly different in the case of fruit trees as, with transgenic papaya, there has already been one example of a fruit from a transgenic tree-like plant commercialised in North America and apparently in the process of being developed for other parts of the world with field trials being conducted in several tropical countries. In a European context, however, the low acceptance of transgenic products will not allow commercialisation.

More promising for commercialisation in the wider world appears the development of completely new traits to deliver new products and services,

such as the production of pharmaceutical proteins in rubber trees or phytoremediation. However, this is not likely to occur in the near future either, as all work into this direction is as yet still in a very early state of development.

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2 Transgenic Forest Trees in China

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2.1 Introduction

Over the past 15 years there has been remarkable progress in the field of biotechnology worldwide. Chinese agricultural, horticultural and forestal research has employed biotechnical methods very successfully (Huang et al. 2004). However, the Western world has not always fully realised the extent of these developments.

This chapter aims to provide a short review of research in – and use of – genetically modified trees in China, with the main focus on forest trees and on work that developed in a bilateral collaboration between groups in China (Beijing, Baoding) and Germany (Waldsieversdorf) for more than ten years. For the authors this is a first attempt to evaluate the present situation in such a large country. Addressed are also some facts concerning aspects of rules and regulations for this field of research and its subsequent commercial use. This only depicts the present situation, as the process of elaboration of rules and regulations is still going on in China, as it is in other countries (Wang 2004). Some results from Taiwan concerning transgenic trees are also included.

After first successes with agricultural plants, modern methods of biotechnology, including genetic engineering, were extended by Chinese researchers to woody species to shorten the time needed for breeding. Although the classical breeding programmes have been continued, a new focus was placed on the introduction of molecular methods. A IUFRO workshop "*Advances in tree development control and biotechnique*" held in 1993 in Beijing summarized the state of the art in China at that time (Wang 1993).

Some of the main problems confronting Chinese agriculture and forestry are insect pests, plant diseases and environmental stress such as, e.g., drought (Xiang et al. 1995) and locally high soil salinity. Also there was – and still is – an urgent need to satisfy the country's increasing demand for wood and wood products. China is now, according to an FAO paper, the largest importer of

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industrial logs and the second largest importer of forest products in the world (Lu 2004). At the same time, the protection of natural forests, which had become necessary because of the severe ecological impact of deforestation in the past, contributed to a shortage in wood production. Therefore considerable afforestation programmes were started, which, apart from wood production, aimed at different purposes, such as the protection of large areas against desertification. These afforestation programmes required high quality plant material (Weisgerber et al. 1995). Right from the beginning a central element in these programmes was breeding. It was initially aimed to increase the growth rate in trees and later on focused on wood improvement (Han 1995). Besides conventional breeding methods, the transfer of foreign genes into trees offered a chance to improve the tree crop while shortening breeding periods. Especially fast growing broadleaved tree species such as poplar and eucalyptus were the target species for gene transfer. These species are mainly propagated vegetatively and used for shorter rotation periods. In some cases difficulties in vegetative propagation of poplar were overcome by an improved rooting behaviour after an *Agrobacterium rhizogenes* transformation (Zheng et al. 1995). One of the first transformations in poplar in China with the help of *Agrobacterium* was reported by Wang et al. (1990).

2.2 Production of Insect Resistant Transgenic Forest Trees in China

Insect attacks and diseases are the main factors for economic losses in forestry (see also Chap. 12). According to incomplete statistics dating back to the 1950s, the 1960s and the 1990s, an annual increase of losses of 25% was calculated (Su et al. 2003). The present forest covers an area of 133 million hectares with the national forest cover rate of 13.92%. An area of 8.2% of the total forest area and 23.7% of the total plantation area of China was affected by insect pests or diseases, the economic loss reached more than 5 billion RMB. These factors have to be kept in mind when sustainable forestry is the aim of afforestation projects.

Insect pests are one of the main problems for forestry in China. This applies especially to plantations where severe losses are observed and the insects are often the limiting factor for tree growth. The main targets of insect attacks on trees are the leaves and the trunk. The insects damaging the leaves are easier to get under control than the insect larvae living inside the trunk. The control of these pests with insecticides has detrimental ecological effects and can only be a useful method on a smaller scale such as in nurseries. Knowledge on biological pest control using different insecticide proteins from a variety of *Bacillus thuringiensis* (Bt) strains was the basis for attempts to express genes coding for these proteins in crop plants themselves. Agricultural crops transformed with the so-called Bt genes were the first

target plants (e.g. maize, soybean and cotton) and there already is an extensive commercial use of such plants worldwide. The first successful transfer of Bt genes into poplar trees in China was carried out by Wu and Fan (1991), followed by the groups of Tian and Han (Tian et al. 1993, 1995; Chen et al. 1995a,b). These Bt genes or modified parts of Bt genes aimed at killing the main defoliator insects. Starting with *Populus nigra* plants, several other poplar species were transformed in due course (Chen et al. 1995c; Wang et al. 1996, 1997; Hao et al. 1999; Tian et al. 2000). For transgenic poplar lines expressing Bt, a change in leaf morphology and growth was observed (Wang et al. 1996, 1997). This resulted in a reduction of the number of transgenic lines available for a possible practical application. A review of work on genetic transformation to create forest trees with enhanced insect resistance was presented by Wang LD et al. (2004). This paper summarised the results of some of the first field trials with Bt poplar.

In search of suitable candidate genes to achieve resistance against one of the main pests in poplar (*Clostera anachoreta* F.), different proteinase inhibitors were tested (AHTI-Arrowhead trypsin inhibitor, AMTI-2 *Allocasia macrorhiza* (giant taro) trypsin inhibitor 2, BBI – Bowman-Birk inhibitor, CETI – chicken egg trypsin inhibitor, CMTI-1 – *Cucurbita maxima* trypsin inhibitor, POT-1 – potato proteinase inhibitor and many others, Bian et al. 1995). Pumpkin fruit trypsin inhibitor and potato proteinase inhibitor 1 were the most effective inhibitors in this study.

A protein which was used to transform poplar plants was ABP-LC1 (antibacterial polypeptide LC1, Li et al. 1996) from *Bacillus subtilis* strain A014 (Zhu et al. 2001). This protein was originally described as an antibacterial protein active against the causal agents of diseases like rice leaf blight (*Xanthomonas campestris pv. oryzae*). The development of insects was also inhibited by this protein. It was reported by the authors that upon feeding on transgenic poplars expressing this protein the mortality of the Asian longhorn beetle (*Anoplophora glabripennis*) was more than 75%.

Building on initial work on the transfer of single genes into poplar, advanced experiments aimed at the transformation with plasmids containing a combination of two genes, which code for proteins that are able to inhibit insect development, were carried out (Li ML et al. 2000). Using a binary vector carrying a partially modified BtCry1Ac gene fused to an arrowhead (*Sagittaria sagittifolia* L.) proteinase inhibitor gene (API) several transgenic lines could be generated (Tian et al. 2000; Zheng et al. 2000; Yang et al. 2003). Such an approach (the use of two genes inhibiting insect development in different ways) might help to prevent the formation of resistances in pests against insecticide proteins, which have already been shown in the laboratory (Fox 2003).

For these experiments a poplar (hybridclone 741) created in 1974 was used. This hybrid is a complex cross of several poplars [*Populus alba* L. × (*P. davidiana* Dode + *P. simonii* Carr.) × *P. tomentosa* Carr.]. The fast-growing female hybrid can produce flowers, but the formation of seeds is restricted and these seeds are not able to germinate under natural conditions. As

therefore the risk of unwanted gene flow of transgenes into the environment and natural populations is reduced, this hybrid was considered ideal for transformation work.

Other genes were also used for these transformations, such as a lectin gene from snowdrop (GNA1 – *Galanthus nivalis* agglutinin) and a BtCry3 gene specific against beetles. Experiments in nurseries and in field trials confirmed the lasting influence of feeding of these transgenic plants (BtCry1Ac + API) on insect growth. The effects of these transgenic lines using a combination of two genes on some of the main defoliating insects in North China were tested and the results published by Yang et al. (2003). Bioassays on larvae of Gypsy moth (*Lymantria dispar* L.), Scarce Chocolatetip (*Clostera anachoreta* F.), Fall webworm (*Hyphantria cunea* D.), Vapourer moth (*Orgyia antiqua* L.) and *Micromelalopha troglodyta* (G.) showed that three transgenic clones had a high resistance to these insects but the insecticidal activity was different in different years and for different insect species within the observation period of four years. The insect resistance of these transgenic lines (BtCry1Ac + API) was also dependant on ambient temperature (Liu et al. 2004a). Both, an increase in total mortality and accumulative mortality of Gypsy moth (*Lymantria dispar* L.) was positively correlated to a rise in temperature. This effect is likely to be linked to the fact that on the one hand a higher temperature accelerated the development of the larvae on leaves of the control plants and on the other hand the sensitivity to the toxin decreased with increasing number of instars. Also, *Clostera anachoreta* F. showed a temperature dependence of mortality on high and medium resistant transgenic clones.

In other test series, larvae of Gypsy moth (*Lymantria dispar* L.) and *Clostera anachoreta* F. were fed indoors (Liu et al. 2004b). The effects over several insect generations were of interest. The mortality of larvae fed on insect-resistant poplar for two generations was higher than for larvae fed on the same plants for one generation. The mortality of larvae fed on insect resistant poplar in a former generation and fed on control trees during the next generation was higher than for larvae fed on the control trees for two generations. The exuviations index, quantity of feeding and the rate of pupa and larvae fed for two generations on insect-resistant transgenic trees were lower than those fed for one generation on insect-resistant clones and afterwards on control plant leaves or for two generations on control leaves. These results indicate that the resistance which is based on two genes (BtCry1Ac + API) is sustainable and led to an increase in larvae mortality and a decrease in the density of next generation larvae. This also included a retarded larvae development in Gypsy moth. By using the method of inoculating insects in a coop set on a tree it was shown that, from 18 transgenic clones tested, 4 clones could resist the long-horned beetle *Apriona germari* (Hope) and clone influenced the insects only marginally (Wang et al. 2002). Three of the resistant clones contained a BtCry3 gene whereas the fourth resistant clone and a further one with lower resistance contained a GNA1 (agglutinin) gene.

In 2003 and 2004 laboratory feeding tests against leaf beetles were carried out in Germany with 16 transgenic lines based on poplar hybrid clone 741 as a part of bilateral cooperation. These lines were the same ones described above (seven lines with BtCry1Ac + API; three lines with BtCry1; five lines with BtCry3 ,one line with GNA1). The experiments confirmed the stability and specificity of the inserted genes on the growth of insect larvae tested. Only transgenic plants with inserted BtCry3 gene inhibited the growth of poplar leaf beetle larvae (*Melasoma populi* L. formerly *Crysomela populi* L.) and imported willow leaf beetle larvae (*Plagiodera versicolora* LAICH.) completely. These are the most common damaging insects of poplar in Germany. On the other hand, a leaf-eating sawfly (*Stauronematus compressicornis* F.) was not influenced at all by any of the inserted genes.

In China recent experiments were also carried out to generate transgenic trees whose tissue, if eaten, would cause the inhibition of trypsin as the major hydrolytic enzyme during digestion processes in insects. Inhibition of this proteinase by proteinase inhibitors has severe effects on insect growth. Cowpea trypsin inhibitor (CpTI), a small polypeptide, was transferred into poplar [(*Populus tomentosa* × *P. bolleana)* × *P. tomentosa*]. The results revealed an enhanced resistance to three main insects (*Malacosoma disstria* L., *Lymantria dispar* L. and *Stilpnotia candida* Staudinger). The growth parameters of five transgenic lines were not negatively influenced compared with the control after one year (Zhang Q et al. 2002). Quite the opposite was the case as height and basal diameter of one transgenic line (TG04) was significantly increased. Three clones were characterized by a higher insect resistance. It was confirmed in field trials that this gene was still present in the transgenic trees after two years (Zhang Q et al. 2004).

The authors stressed that, particularly when working with genes such as the ones mentioned above, which are not as specific for a particular group of target organisms as Bt genes, it is important to carry out studies on biological safety concerning their potential threat to other living organisms present in the environment. A successful transformation of poplar with Bt genes in combination with CpTI was reported by Guo et al. (2004).

The genetic transformation of birch (*Betula platyphylla* SUKACHEV) was published by Zhan et al. (2003). This group used a chimeric gene constructed from a spider peptide gene causing insect resistance fused to a C peptide sequence of a Bt gene).

First feeding experiments showed that the development of Gypsy moth was retarded. Among other insecticide genes which were used to influence the insect growth was the AaIT gene from a scorpion (*Androctonus australis*). This gene was transferred into a hybrid poplar (clone N-106; *Populus deltoides* × *P. simonii*) and causes the formation of an insect-specific neurotoxin. Sixty-two transgenic plants were regenerated and among them one line "A5" was significantly resistant to feeding by first instars larvae of Gypsy moth in comparison with the control plant (Wu et al. 2000).

Recent results showed that the search for new Bt genes which are toxic to stem-boring beetles can be successful. A *Bacillus thuringiensis* strain (Bt866) was isolated from a dead beetle. This strain formed a Cry3Aa-like gene (Bt866cry3Aa) which is highly similar to Cry3Aa1 and caused a high mortality on the images of beetles (Chen et al. 2005). The gene was expressed in *E. coli*. When bacterial solution was mixed with artificial feed it resulted in a high-level toxicity towards *Apriona germari* Hope larvae. Now the construction of a binary vector containing this gene for plant transformation is underway. Strategies against trunk damaging insects (e.g. *Anoplophora glabripennis*) are very important for current as well as for future afforestation projects in China. The Three North Shelterbelts Project, which will cover a very large area (approximately 35.08 million ha) when finished, is already under threat by insect attacks, mainly by the Asian longhorn beetle. The reduction in timber output in Chinese forestry due to pests has been estimated at approximately 17 million cubic metres which goes with a high economic loss. A spread of these insects from plantations into natural forests is possible (Su et al. 2003). Therefore there is an urgent interest in overcoming the problem and biotechnology offers a real and fast solution.

2.3 Transgenic Trees Tolerant to Environmental Stresses

Right from the beginning of tree genetic engineering some of the main problems that were tackled were related to environmental stress. Drought, frost and local salinity are stress factors that are central problems in forestry in China. Therefore the first experiments were aimed at alterations in metabolism which allowed the plants to adapt to stress conditions. Initial work was carried out by Liu et al. (2000) who transferred a gene from *E. coli* (mannitol dehydrogenase, mtl-D) into poplar (*Populus* × *Xiaozhannica* cv. "Balizuangyang"). The transgenic plants recovered from this work were reported to grow well on a nutrient medium with 0.6% (w/v) sodium chloride whereas control plants died already at 0.4%. A later report by Sun et al. (2002) showed that the root formation and structure of these transgenic plants was improved (more top roots, side roots and higher root length) compared with the control under the same salt stress. Meanwhile this transgenic poplar was registered for the protection of breeder's right under the name Taiqqing No.1 officially and is used at a larger scale (Wang 2004). Transgenic Balizhuangyang poplar trees had not only a higher tolerance to salt but also a vigorous growth and better performance. It was concluded from statistical calculations that the limit of salt tolerance for this clone is 4.3 g salt/kg soil (0.43%). PCR could confirm the stable integration of the gene in the trees included in this field experiment. No gene loss occurred (Yin et al. 2004).

A combination of mtl-D gene with gutD gene from *E. coli* (encoding glucitol 6-phosphate dehydrogenase) was introduced in poplar (*Populus*

deltoides × *P. cathayana*; Fan et al. 2002). Integration and expression of genes was shown. The authors mentioned that a clearly enhanced salt tolerance could be shown. In addition, genes of enzymes related to the synthesis of glycine betaine were introduced into poplar trees to cause a salt tolerance of the plant (Yang et al. 2001). The results showed that the Bet-A gene (encoding choline dehydrogenase, synthesizing glycine betaine) from *E. coli* was integrated in nuclear DNA in the leaves of poplar (*Populus simonii* × *Populus nigra*).

Another strategy to improve salt tolerance was the transformation of *Populus deltoides* with an antisense phospholipase Dγ gene (Anti-PLDγ; PLDγ from *Arabidopsis thaliana*; Zou et al. 2004). This gene was introduced by Agrobacterium transformation into a poplar clone (G2). Twenty-one kanamycin resistant plants were obtained after successive selection during shoot and root formation. Detection based on PCR and Southern blot confirmed 13 of these plants to be positive transgenics. The successful integration led to a positive tolerance to salt for 4 of the 13 plants which authors described as "enhanced to a certain extent".

The introduction of genes linked to ethylene biosynthesis led to an influence on ethylene production and therefore also on senescence and a prolonged growth (Li et al. 1999a,b). The genes for ACC (1-aminocyclopropane-1-carboxylic) synthase from soybean and ACC oxidase from tomato which code for enzymes that catalyse initial steps in ethylene biosynthesis were introduced in antisense direction in *Populus deltoides*. This resulted in 'silencing' of the homology of these genes in the plant and a reduction of the ethylene production. When a plasmid with a combination of the two genes in antisense orientation was used for transformation the ethylene production decreased to one-tenth of control plants (Li Dissertation, not yet published). This was a remarkable effect and an improvement of gene action in comparison with the introduction of the single genes.

2.4 Sterile Transgenic Forest Trees

One of the main problems for the use of transgenic trees from a practical point of view is to prevent an uncontrolled escape into the environment. Two ways for the spread of transgenes into a natural environment are possible. One way is by root suckers. Some of the trees which are used today also show a high capability to form root suckers, e.g. poplar, eucalypts, black locust, etc. This problem can be overcome by the separation of plantations with transgenic trees from natural stands and the surrounding area of the plantations can be treated with herbicides to stop an escape by root suckers.

The more serious problem is the possibility that pollen and seeds can be spread by wind over longer distances and the transgenes can enter natural stands and invade into indigenous populations. This problem can be tackled

by using hybrids like clone 741, which can form only female flowers, but no fertile seeds. For other tree species, which are in the focus to be transformed with genes of economical interest, such sterile clones are often not available. Therefore there are increasing research efforts to develop transgenic plants which can form flowers but no fertile pollen or seeds. Work carried out by Chinese scientists to solve this problem includes experiments with TA29-barnase fusion constructs. TA29-barnase is a fusion product of the TA29 promoter from tobacco that is expressed specifically in anthers and the barnase gene from *Bacillus amyloquefaciens*, which codes for a ribonuclease that degrades RNA. Hence this gene works as a "terminator" gene. This TA29-barnase gene, causing male sterility, was introduced by particle gun into already transformed plants carrying Bt genes (Li L et al. 2000). Further observations, after these plants have reached maturity, will show if the formation of pollen is inhibited.

2.5 Further Transformation Work on Forest Trees

Some work was also done on the creation of disease-tolerant trees. It was, however, on a much smaller scale than the work on insect tolerance. The Forestry University in Beijing has carried out work in breeding *Eucalyptus urophylla* ST BLAKE lines with an increased tolerance for *Pseudomonas solanacearum*. The basis for that transformation was the introduction of the cecropin D gene which codes for an antibacterial peptide into the plant (Shao et al. 2002). Such transgenic plants were less affected by inoculation with *Pseudomonas solanacearum* compared to control plants of the wildtype.

Work is also underway to transform poplar plants with genes which can cause resistance to fungal attack. A research group of Shandong Agricultural University developed a regeneration system and transformed *Populus deltoides* clone G1 with the chitinase 5B gene (CH5B-from French bean *Phaseolus vulgaris*). The integration of the gene into the plant genome was confirmed (Meng et al. 2004). With the same poplar clone (G1) a glucanase gene (β-1,3-glucanase; BG2 was also introduced and the integration of the gene into the plant genome has already been confirmed (Han et al. 2004).

Investigations aimed at reducing lignin content in poplar wood were carried out at the Beijing Agro-Biotechnology Research Center. The antisense gene of 4-coumarate CoA ligase (4CL) was transformed using *Agrobacterium tumefaciens* into a triploid white poplar (*Populus tomentosa* Carr.; Jia et al. 2004). The integration of the gene was confirmed by PCR and Southern blotting. RT-PCR and Western blot showed the expression of the gene. Transgenic poplars which were obtained had a reduced lignin content, but were unchanged in their holocellulose content. The wildtype clone used for this work is a rapid growing tree that has been bred for afforestation and as

raw material for the pulp and paper industry. The growth rate was unchanged in transgenic individuals.

A Taiwanese publication reports a successful transformation of *Eucalyptus camaldulensis* with the reporter genes GUS (g-glucuronidase) and NPT (neomycin phosphotransferase) by *Agrobacterium tumefaciens* transformation (Ho et al. 1998). This kind of work was the necessary precondition for the transfer of the cinnamate 4-hydroxylase gene (C4H) from *Populus tremuloides* L. (Chen et al. 2001). This gene was inserted in sense or antisense direction. In comparison with control shoots and transgenic shoots with the ß-glucuronidase gene the transgenic shoots with C4H proliferated and elongated faster. More than 100 plants were produced for further characterization concerning a possible alteration of lignin composition or lignin content. A group of researchers from the Chinese Academy of Sciences (Wei et al. 2001) transformed a hybrid of *Populus tremula* × *P. alba* with an antisense gene of caffeoyl CoA *O*-methyltransferase (CCoAOMT) from the Chinese white poplar (*P. tomentosa*). The measurement of the lignin content in five- to six-month-old transgenic plants showed that one transgenic line had a lower lignin content (17.9% less) as compared to the control. Although the authors highlighted the benefits they also addressed possible consequences for the stability and resistance of the plants as well, given that lignin is central to wood structure and plays a role in plant defence.

2.6 Field Tests of Transgenic Trees

One of the first field trials with transgenic poplars (*P. nigra*) was established in China at Manas Forest Station, Xinjiang Uygur Autonomous Region (Fig. 2.1).

The evaluation of this field trial was carried out in different time intervals, e.g. by Hu et al. (1999, 2001). Results showed that the amount of leaves with severe damage was reduced to 10% in comparison to non-transgenic control plantations nearby where higher damage rates of leaves (80–90%) were observed. The density of pupae in the soil of the plantation with transgenic trees was one-fifth of the density found in non-transgenic control stands. Some results obtained several years ago concerning the transformation of plant material and this field trial in Xinjiang province (Xinjiang Uygur Autonomous Region) have already been published by Ewald and Han (2000).

The transfer of the BtCryIAc-gene and the protection of the leaves from insects resulted also in an increased performance of the trees. A comparison of the breast height diameter of a control tree in the nursery of Forest Academy in Beijing with an isogenic transformed tree showed an increase of diameter of approximately 10 cm within seven years of planting (in 1993) under optimal conditions (i.e. 17.5 cm vs 27.3 cm). The final diameter of this transgenic tree was 30 cm when it was cut in 2001. The growth parameters of

Fig. 2.1. Field trial with transgenic poplar trees in Xinjiang in spring 2005. Plants are 11 years old

transgenic poplar trees in the first field trial in Xinjiang province are presented in Table 2.1 to underline the impression of actual growth depicted in Fig. 2.1. Information concerning changes in morphology, the performance of four clones and the influence on insect distribution were already given in detail by Wang LD et al. (2004). Some aspects of biosafety were reported in this chapter of Wang LD et al. (2004) as well. Growth parameters of different transgenic lines were compared with untransformed clones of *P. nigra* and *P.* × *euramericana* "Robusta" which were used as control material. Some clones were not confirmed to be transformed (13, 254) and others did not perform like the control (e.g. 222, 139); there was, however, a higher stable performance of some clones compared with the control over several years (12, 153, 172).

This experimental field trial with transgenic Bt poplar in China was not only the first one established to select clones which are suitable for a practical use but also to carry out necessary investigations concerning biosafety issues (influence on damaging insects, stability of performance and transgenic behaviour, etc.) over a longer time span. According to Wang (2004), the first environmental release of these transgenic trees (*P. nigra* + *Bt*) was in 1998, after approval by the Bio-Engineering Security Committee of the

Table 2.1. Growth parameters of transgenic poplar clones in the first experimental field trial in Xinjiang. The trial was established with two-year old plants and measured in spring 2003 when plants were nine years old (data were kindly provided by Hu JJ)

Clone No.	DBH (cm)	Std. error	Height (m)	Std. error
12	**25.2**	**0.871**	**18.3**	**0.64**
208	23.1	0.946	16.9	0.695
209	22.6	1.152	16.6	0.847
153	**22.4**	**0.871**	**17.6**	**0.64**
172	**22.2**	**0.795**	**17.9**	**0.584**
P. × euramericana 'Robusta'	22.2	0.798	16.7	0.587
Control 2	21.6	0.718	18.2	0.528
13	*20.3*	*0.954*	*16.8*	*0.701*
254	17.3	0.871	14.2	0.64
Control 3	15.6	0.914	13.4	0.671
110	15.5	0.954	14.1	0.701
197	15.2	0.842	12.6	0.619
141	15.1	0.914	14	0.671
192	14.8	1.742	11.2	1.28
162	14.7	0.871	12.8	0.64
222	14.3	1.35	12	0.992
139	12.6	0.914	11.3	0.671

DBH – diameter at breast height, best performing transgenic lines are printed in bold letters
Non-transgenic controls: *P. × euramericana* 'Robusta', *Populus nigra* clones: Control 2, Control 3
Lines: 12, 208, 209, 153, 172, 13, 254, 110, 197, 141, 192, 162, 222, 139, but not lines 254 and 13 were confirmed to be transgenic by PCR

Ministry of Agriculture. So-called pilot plantations were established in six provinces (Beijing, Jilin, Shandong, Jiangsu, Henan and Shanxi).

Over the last years several other field trials with transgenic poplar trees were established. There are two field trials in Huairou near Beijing where transgenic and control trees were planted in mixture. Three transgenic *Populus nigra* clones and one transgenic *P. deltoides* clone will be tested in these field trial. This plantation has a well which supplies water individually to all plants by an underground tubing system. The area of this field trial is 33 ha (Fig. 2.2). There is a second field trial in Huairou close to the first one but without an irrigation system. A larger field trial was established in spring 2004 in Liaoning province not far from the city Huludao. The area of the test field trial with transgenic trees is also 33 ha within a total plantation area of 466 ha which will be further extended in the future. Transgenic trees will also

Fig. 2.2. Field trial with transgenic and control poplar trees in Huairou in 2004

be grown on the commercial managed plantation area. This field trial contains different mixtures of transgenic and control trees. To elaborate optimal planting systems with reduced damages performance and the influence on insect distribution will be studied.

Experiments of researchers to confirm the stable insertion of transferred genes after four and eight years within these plants showed the presence of the Npt II and Bt genes (Wang JH et al. 2004). These results confirmed the stability of these transferred genes in the poplar genome. The authors also carried out pollinations with a male transgenic tree under controlled conditions. In the progeny a 1:1 Mendelian segregation of the gene was found. In order to study the effects of transgenic forest trees on the environment, in the context of investigations aiming to evaluate biosafety, soil microorganisms were determined in the soil around the roots of control and transgenic trees (Hu et al. 2004). Three groups of organisms were included in the survey: bacteria, actinomycetes and mould. The analysis which was carried out and evaluated using ANOVA showed that there were no significant differences in density of associated soil microorganisms among both the individual trees and the plantations. This indicates that there are no significant negative effects of transgenic poplar on the soil microbial ecosystems.

Effects of transgenic poplars with Bt CryIAc gene on the structure of the insect communities were investigated and the results presented previously at

the Seventh International Symposium on the Biosafety of Genetically Modified Organisms in Beijing in 2002 (Zhang Z et al. 2002) and published subsequently (Zhang Z et al. 2004). The study came to the conclusion that the insect and spider community was not significantly different in smaller test plots but in larger test plots there was a significant difference. In a comparison of a pure transgenic plot vs a 1:1 mixture of transgenic and control plants, the amount of the main lepidopterous pest (*Clostera anachoreta* [Fabricius]) was reduced in the transgenic plot and the main defoliating insect became a poplar sawfly (*Pristiphora beijingensis*). The density of ladybirds (individuals/twig) was ten times higher in the mixed transgenic poplar stand compared to the pure transgenic poplar stand (averagely 0.21/twig vs 0.021/twig) whereas the density of spiders was higher in the pure transgenic stand (averagely 0.125/twig and 0.0625/twig, respectively). Although the damage rate attributed to insects in mixed stand was somewhat higher (18.48%) compared with the pure transgenic stand (11.26%), it was not significant. From these results the authors (Zhang Z et al. 2002, 2004) concluded that more research is necessary to get sufficient information about insect distribution in fields with different mixtures of transgenic and wildtype clones.

In the province of Hebei, field trials with transgenic trees were established by the Agricultural University using the hybrid poplar 741 containing two genes which influence insect development (Bt and API). The results showed that the foreign genes remained stably integrated within the plants since 1997 (Yang et al. 2003). First investigations in field trials with these plants compared control plots with transgenic trees of the same clone (741). The transgenic trees showed differences in insect resistance between each other as well as in comparison with control trees. After a five-year experiment the Forest Bio-Engineering Security Committee approved the field release of six transgenic lines of poplar clone 741 in 2001.

Investigations concerning the occurrence and distribution of insects in transgenic and control poplar plots were carried out by Gao et al. (2003). As the authors mentioned, the presence of transgenic poplar can reduce the density of individuals of defoliating insects and shift the dominance of individual species. On the other hand, the insect diversity was enhanced, especially within test plots of transgenic 741 trees with a lower insect resistance (Gao et al. 2003). This is in accordance with positive influence of transgenic plants on insect diversity that was already observed from large-scale investigations in transgenic cotton in 2000–2001 in Northern China (Jia and Peng 2002). Nevertheless it became obvious in some field trials when these transgenic trees (poplar 741+Bt+API) were damaged by the Asian longhorn beetle (*Anoplophora glabripennis*) that the application of transgenic trees is unable to overcome all insect problems at once.

Field tests were carried out in Tianjin City and Shandong Province with the salt-tolerant poplar clone Taiqing No.1 (former *Populus* × *Xiao Zhannica cv.* "*Balizhuangyang*" given another Chinese common name in December 2003: Zhongtianyang [*Populus* × *xiaozhuanica* W.Y. Hsu et Liang cv.

'*Balizhuangyang-zhongtian*']) resulting from the work of Liu et al. (2000) and Sun et al. (2002). The size of the field trial in Shandong was 6.6 ha. The results confirmed that the survival rate of transgenic poplar was significantly higher compared with control plants on soil containing 0.3–0.4% salt. After being approved for field release this salt-tolerant clone will be planted in Shandong province in areas close to the sea where saline soils exist (Wang 2004).

2.7 Commercial Use of Transgenic Forest Trees

At present, in the majority of cases, research work with transgenic trees is at the stage of laboratory experiments or nursery tests as shown in Table 2.2. In 2002 several poplar clones were approved by the Gene Security Committee of the State Forestry Administration for commercial use after the required field testing. Some clones of transgenic forest trees were created years ago and therefore have already been tested on a larger scale in field trials, some of them also in different provinces under different conditions. These transgenic trees have demonstrated a high growth performance and a lasting effect of the transgene against the main target insects over several years. Included in the approval for commercial use were the first clones of *Populus nigra* trees which contained a modified Bt gene and which were tolerant against *Lymantria dispar* and other lepidopterous insects. Also three clones of the poplar hybrid 741 with a combination of two genes (Bt and API) were accepted for a commercial use.

Transgenic trees of *Populus nigra* from the Chinese Forestry Academy and transgenic lines of the hybrid poplar tree 741 from the Agricultural University Hebei are thus at present the only two transgenic forest trees approved for commercial use in China. For this reason some field trials were already established and supported by commercial growers. The above-mentioned field trial with different transgenic poplar clones in Hulodao is aimed at the elaboration of results concerning optimal planting structures and a production of trees with an estimated rotation time of ten years. In the studies carried out in these experimental plantations both effects of fertilization and a combination with agroforestry will be included. That means that, between the rows, agricultural crops such as groundnut (*Arachis hypogea* L.) will be planted. It was indicated by Prof. Han and the local owners of the plantation during a visit in 2004 that the wood from these plantations will be harvested mainly for plywood production. In other parts of China there is also a strong interest in industry to use improved poplar material which includes both, transgenic and non-transgenic trees improved by conventional breeding, such as the poplar clone "Beikang" with an enhanced resistance against the Asian longhorn beetle.

The high likelihood of an increase in the commercialization of transgenic forest trees in China is obvious. The demand for wood and the need to meet different cultivation challenges such as insect damage, drought, salt and others

Table 2.2. Research progress of transgenic forest trees in China (data related to tree numbers refer to publications or information provided by the authors)

Institution	Tree species	Target traits	Gene transferred	Year of trans-formation	Year of first field trial	Spread under controlled conditions	Number of plants raised	Size of field trials	References
Chinese Forestry Academy of Sciences	*Populus nigra*	Insect resistance	BtCry1Ac	1993	1994	2002 (approval for commercial use)	1 Million	66 ha	Tian et al. 1993 Wang et al. 1996 Hu et al. 2001 Wang LD et al. 2004
Agricultural University of Hebei	*Populus alba* L. × *P. davidiana* Dode + ×*P. tomentosa* Carr.) hybrid "741"	Insect resistance	BtCry1Ac +API-A (Arrowhead Proteinase Inhibitor-A gene)	1996	2000	2002 (approval for commercial use)	0.4 Million	20 ha	Tian et al. 2000 Zheng et al. 2000

(*continued*)

Table 2.2. (*continued*)

Institution	Tree species	Target traits	Gene transferred	Year of trans-formation	Year of first field trial	Spread under controlled conditions	Number of plants raised	Size of field trials	References
Bio-technology Research Centre, CAAS, Beijing	Clone N-106 (*P. deltoides* × *P. simonii*)	Insect resistance	AaIT gene neurotoxin	2000 oxin			62		Wu et al. 2000
Taiwan Forestry Research Institute	*Eucalyptus camaldulensis*	lignin content	GUS *Populus tremuloides* – C4H (cinnamate 4-hydroxylase gene)	2001			100 rooted cuttings		Ho et al. 1998 Chen et al. 2001
Institute of Genetics, Chinese Academy of Sciences and Shandong Agriculture University	"Balizh-uangyang" (Populus hybrid)	salt tolerance	Mtl-D (1- phosphate mannitol dehydro genase gene)	2000	2002	2004	6.6 ha		Liu et al. 2000 Sun et al. 2000

stimulate the influence of biotechnology in forest tree breeding as well as in other fields working with trees.

2.8 Rules and Regulations

Right from the beginning of the work with transgenic plants the Chinese government was interested in establishing rules and a regulatory framework, including forest trees because of their potential influence on the environment and human health. General aspects of biosafety research in China concerning GMOs were reported in a review article by Jia and Peng (2002). In accordance with international regulations there are different steps in the development towards the establishment and the use of GMO trees: laboratory work, field tests, environmental release and finally the approval for commercial use. The "*Biosafety administration regulation on genetic engineering*" was already published and confirmed in December 1993 by the Chinese State Science and Technology Commission (SSTC; Huang and Wang 2002). In July 1996 the Ministry of Agriculture (MOA) has put the "*Biosafety administration implementation regulation on agricultural biological genetical engineering*" into practice. The release of transgenic poplar plants to six sites (approximately 300 ha) in 1997 and 1999 was a consequence of the decision to permit the release of transgenic trees by the Bio-Engineering Committee of the Chinese Agricultural Ministry.

In May 2001 the State Department of China legislated the "*Safety administration regulation on agricultural gene modified organism*". These regulations fixed guidelines for agricultural transgenic products. As matching measures in March 2002 the "*Biosafety evaluation and administration measures on agricultural genetically modified organism*", "*Biosafety administration measures on import of agricultural genetically modified organism*" and "*Administration measures on marking of agricultural genetically modified organism*" passed the Ministry of Agriculture. The policy measures related to biotechnology, especially in agriculture, had already been presented in more detail by other authors (Huang and Wang 2002; Marchant et al. 2002). The approval of transgenic poplar clones for the first commercial use in 2002 was the final step in the evaluation process for the plant material described above. Nevertheless, a broad variety of experimental designs concerning biosafety and risk assessment research will be carried out additionally in the future to study the ongoing influence of transgenic trees on the environment over longer time periods (Lu et al. 1999).

2.9 Conclusions

Genetic engineering is becoming a routine method in agriculture and forestry. Although the possibilities in agriculture are much better because of

the broader knowledge available and background breeding information, forest trees are also clearly in the focus of research. The ability to transfer genes into the genome of trees offers ample opportunities in the field of breeding research. Based on the Chinese government's aim to enlarge the total area covered by forests to 19% by 2010 and to 23% by 2020, biotechnology is going to play a central role to tackle specific challenges. Trees are long living organisms and often surrounded by their wild relatives, which causes a risk of gene flow from transgenic stands into natural populations. Therefore, there are additional important aspects of research compared with agricultural crops. Research is still needed to investigate the problems related to transgenic trees, e.g. concerning all aspects of biosafety including efforts to prevent the escape of transgenes into natural populations. Approval for the commercial use of transgenic trees and their easy vegetative propagation by cuttings (e.g. in poplar) can cause a rapid distribution of transgenic plant material in the near future. However, it is also worth mentioning that the awareness of biosafety and risk assessment research is increasing in China too. This can help one to use the transgenic material wisely according to the recommendations which scientists have elaborated and are still elaborating to avoid negative effects like adaptation of insects (tolerance or resistance) by uncontrolled planting. Because of the increased use of GMOs in agriculture and forestry in China it was important that the 7th International Symposium on the biosafety of genetically modified organisms was held in 2002 in Beijing (http://www.isbr.info/document/isbgmo.pdf). Some of the urgent problems concerning the use of GMOs, including trees, were already stressed there.

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3 Modification of Perennial Fruit Trees

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3.1 Introduction

Genetic transformation provides the means for modifying single horticultural traits in perennial plant cultivars without altering their phenotype. This capability is particularly valuable for perennial plants and tree species in which development of new cultivars is often hampered by their long generation time, high levels of heterozygosity, polyembryony etc. Recombinant DNA technology offers opportunities for widening the available gene pool for fruit improvement; it also allows the introduction of several desirable genes in a single event, and can reduce the time to introgress novel genes into elite backgrounds. Although genome transfer via protoplast fusion can circumvent such problems such as sexual incompatibility, polyembryony, and male or female sterility encountered in conventional sexual crossing, transferring only the desirable gene into one species is difficult and nearly impossible. During protoplast fusion, desirable and undesirable traits in the whole genome of the donor parent-introduced into the recipient partner at the same time. Comparatively, genetic transformation is more targets oriented than the genome transfer.

Transgenic walnut was the first transformed fruit tree that was reported by McGranahan and co-workers in 1988. Since then, transgenic technology has been successfully introduced in other fruit trees such as apple, citrus, pear, grapevine, kiwifruit, peach, plum, avocado, papaya, mango, etc. Various genes have been introduced into fruit crops to improve agronomical traits including enhancing bacterial, viral, pest and environmental stress resistance, increasing storage life span, shortening juvenile stage and improving fruit qualities etc. The most successful case came from papaya (Bau et al. 2004), the transgenic papaya with the papaya ring-spot virus coat protein gene (PRSV-CP) performed well and was permitted to grow in commercial scale in the open field (see Chap. 9). Besides this, the U.S. has allowed approximately 124 confined trials of transgenic trees including fruits; transgenic plum clone C-5 with plum pox virus coat protein gene (PPV-CP) has been

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released into field in Poland (Malinowski et al. 1998) and Romania (Ravelonandro et al. 2002) since 1998.

3.2 General Overview of Transformed Fruit Trees

Attempts to improve crop plants by genetic engineering techniques depend very strongly on the availability of reliable protocols for transformation, selection and regeneration. Optimizing the protocol is essential for genetic transformation. For example, use of proper co-cultivation medium and conditions led to a higher number of stably transformed cells and to an increase in the final number of regenerated transgenic plants. The work of Dominguez et al. (2004) showed that inefficient selection could be attributed to the protection of the non-transformed cells from the selective agent by the surrounding transformed cells, and to the persistence of kanamycin-resistance.

Explants used in fruit tree transformation include internodes, epicotyls, leaf, protoplasts, embryogenic callus, embryos and so on (Table 3.1). The leaf disc method, a very simple and convenient transformation protocol with

Table 3.1. List of transgenic fruit trees with agronomical genes

Fruits or species	Cultivars	Method	Genes	Explant	References
Apple (*Malus pumila* Mill.)	"Galaxy"	Ag	Genes encoding lysozyme (T4L) from T4 bacteriophage, attacin E (attE) from *Hyalophora cecropia* pupae; wheat puroindoline B (pinB) for scab resistance	L	Ko et al. (2000, 2002); Faize et al. (2004)
	"Marshall McIntosh"	Ag	Fungus *Trichoderma atroviride* endochitinase or exochitinase resistant to apple scab	L	Bolar et al. (2000, 2001)
	"Royal Gala"	Ag	Fruit-specific apple PG (Polygalacturonases); avidin or strepavidin for insect resistance; acetolactate synthase gene for herbicide; cecropin MB39 for fire blight resistance; HcrVf2 for scab resistance	L	Yao et al. (1999); Liu et al. (2001); Atkinson et al. (2002); Markwick et al. (2003); Belfanti et al. (2004)

(*continued*)

	"Orin"	Ag	Sorbitol-6-phosphate dehydrogenase (S6PDH) related to salt stress tolerance	L	Kanamaru et al. (2004)
	"Greensleeves"	Ag	Antisense apple ACS (ACC synthase) or ACO (ACC oxidase) to improve fruit storage	L	Dandekar et al. (2004)
	"Holsteiner", "Cox" and "Elstar"	Ag	Stilbene synthase gene (Vst1) from *Vitis vinifera* L. and PGIP from kiwi in apple for antifungal potential	L	Szankowski et al. (2003)
	"Jonegold" and "Fuji"	Ag	Antimicrobial peptides (AMPs) for resistance to fungal, antisense apple PPO; polyphenol oxidase to control browning	L	Broothaerts et al. (2000, 2001); Murata et al. (2000, 2001)
	$M._{9/29}$, $Jork_9$	Ag	*Rol* B genes to increase rooting ability	L	Sedira et al. (2001); Zhu et al. (2001)
	M_{26}, A_2	Ag	*Rol* A gene to reduce tree size; *Arabidopsis* phyB genes to get dwarf phenotype	L	Holefors et al. (1998); Zhu et al. (2001)
	Maruba-kaidou	Ri	*Rol* C gene for dwarfism	L	Igarashi et al. (2002)
Apricot (*Prunus* L.)	*Prunus armeniaca*	Ag	CP-PPV	SE/L	da Câmara Machado et al. (1992, 1995)
Chestnut (*Cerasus avium* L.)	horse chestnut	Ri	*Rol* A, B, C and D genes for root improvement	SE	Zdravkovic-Korac et al. (2003)
	Castanea sativa chestnut	Ag	ß-1, 3-Glucanase (glu) and chitinase (chi) genes exhibit antifungal activity	SS	da Costa Seabra and Pais (1999)
Citrus (*Citrus* L.)	"Duncan" Grapefruit	Ag	RNA-dependent RNA polymerase (RdRp) from CTV; carotenoid biosynthetic genes from grapefruit for metabolic engineering of fruit	E	Costa et al. (2002); Febres et al. (2003)
	sour orange, Mexican lime, "Rio Red"	Ag	CP-CTV; untranslatable coat protein gene (uncp) of CTV and the *Galanthus nivalis* agglutinin gene	E	Ghorbel et al. (2000); Yang et al. (2000);

(*continued*)

Table 3.1. (*continued*)

Fruits or species	Cultivars	Method	Genes	Explant	References
	grapefruit		(gna), p23, p25 of CTV		Dominguez et al. (2002)
	"Valencia"	PEG	Pectin methylesterase gene from *Valencia orange*	P	Guo et al. (2005)
	navel orange, "Early Gold" and "Murcott" tangor	PEG	Citrus canker disease resistant candidate gene Xa21 from rice	P	Guo and Grosser (2004)
	"Ponkan", "Valencia"	Ag	pTA29-barnase gene	EC	Li et al. (2002, 2003)
	"Pineapple" sweet orange	Ag	Tomato pathogenesis related protein PR-5 for antifungal	I	Fagoaga et al. (2001)
	Carrizo citrange, and *Poncirus trifoliata*	Ag	HAL2 for salt tolerance; antisense chilling-inducible ACC synthase gene (CS-ACS1); LEAFY (LFY) or APETALA1 (AP1); p12 for citrus blight (CB); pyrroline-5-carboxylate synthetase mutant gene (p5cs) to increase proline accumulation; citrus FT (CiFT) for early flowering	E	Cervera et al. (2000); Pena et al. (2001); Wong et al. (2001); Kayim et al. (2004); Molinari et al. (2004); Endo et al. (2005)
	West indian Lime	Ag	Genes for decreased seed set	H/E	Koltunow et al. (2000)
Grape (*Vitis vinifera* L.)	"Thompson Seedless"	Ag	Lyric peptide Shiva-1 gene or the tomato ringspot virus (TomRSV) coat protein (CP) gene	SE	Scorza et al. (1996)
	"Gravesac"	Ag/Ri	CP-GCMV (grapevine chrome mosaic virus)	plantlets	Torregrosa and Bouquet (1997)
	Vitis rupestris and 110 "Richter"	Ag	CP-GFLV (grapevine fanleaf nepovirus)	EC/H	Krastanova et al. (1995)
	grape (*Vitis rupestris* S.)	Ag	The movement protein gene of grapevine virus A (GVA)	SE	Martinelli et al. (2002)

(*continued*)

	Neo Muscut, "Chancellor"	Ag/B	Rice chitinase gene (RCC2) to enhance fungal risistance	SE	Kikkert et al. (1996); Yamamoto et al. (2000)
	grapevine root-stocks	Ag	Virus genes and a virE2 gene, Vst1 (*Vitis* stilbene synthase 1) gene	C/SE	Xue et al. (1999); Coutos-Thevenot et al. (2001)
	grapevine Richter 110	Ag	Gene encoding ferritin for stress tolerant; Vr-ERE resistant to toxin	SE	Legrand et al. (2003); Oláh et al. (2004)
Kiwifruit (*Actinidia deliciosa*)	Kiwifruit	PEG	Chloramphenicol acetyl transferase (CAT) gene	P	Oliveira et al. (1991)
	"Hayward"	Ag	*Vitis* stilbene synthase gene for beneficial effects on health; soybean beta 1,3 endoglucanase cDNA for *Botrytis cinerea* resistance	L/S	Nakamura et al. (1999); Kobayashi et al. (2000)
Papaya (*Carica papaya* L.)	"Sunset", R0 clone 55-1	B	CP-PRSV to papaya ringspot virus	SE	Lius et al. (1997)
	"Merida", "Venezuela", "SunUp" and "Rainbow"	Ag	CP-PRSV for papaya ring spot virus resistance	EC	Davis and Ying (2004); Fermin et al. (2004)
	"Tai-nong-2"	Ag	Viral replicase (RP) gene	EC	Chen et al. (2001)
	Papaya	B	PPT (phosphinothricin) resistant	SE	Cabrera-Ponce et al. (1995)
Passion fruit	yellow passion fruit	Ag	Antisense melon ACC oxidase gene (CMe-ACO1) to improve fruit quality	L	Quoirin et al. (2003)
Peach (*Prunus* L.)	"Batsch"	Ag	Cytokinin biosynthesis (ipt) gene	E	Hammer-schlag et al. (1997)
Pear (*Pyrus* L.)	"La France"	Ag	Sense or antisense LF-ACS1 and LF-ACO1 for fruit storage	L	Murayama et al. (2003)
	BP10030	Ag	*Rol* B increased rooting ability	S	Zhu et al. (2003)

(*continued*)

Table 3.1. (*continued*)

Fruits or species	Cultivars	Method	Genes	Explant	References
	"Beurre Bosc"	Ag	*Rol* C gene to improve rooting	L	Bell et al. (1999)
	"Passe Crassane"	Ag	Cecropin B and its analogs (SB-37 and Shiva-1), attacin E for bacterial resistance	L	Chevreau et al. (1999)
	"Passe Crassane"	Ag	Bovine lactoferrin cDNA and EPS-depolymerase for fire blight resistance	L	Malnoy et al. (2003, 2005)
Plum (*Prunus domestica* L.)	plum	Ag	Coat protein gene of plum pox virus (CP-PPV)	H	Scorza et al. (1994); Gonzalez-Padilla et al. (2003)
Walnut (*Juglans* L.)	"Chandler"	Ag	Bt cry1A(c) against insect	SE	Leslie et al. (2001)

Ag: *Agrobacterium*-mediated transformation, B: particle bombardment, C: callus, E: epicotyl, EC: embryogenic cells, H: hypocotyl, I: internode segments, L: Leaf, P: protoplast, PEG: polyethylene glycol mediated transformation, Ri: *Agrobacterium rhizogenes*-mediated transformation; S: stem, SE: somatic embryos, SS: stem segments

rapid plant regeneration process, has become the most common popular means for apple, papaya and other fruit trees. Using mature tissue as the explant is important for the precocious evaluation of the genetically modified characteristic and for maintaining the true-to-type of the variety for those species that their seedlings are from zygotic instead of nucellar embryos. Scientists in Spain explored this technique in the past years; however, the regeneration capacity and the transformation rate are low when the mature tissue is used as the explants, and most researchers still use the juvenile state explants for transformation.

At present, there are four gene delivery methods for fruit tree transformation, including *Agrobacterium*-mediated transformation, polyethylene glycol (PEG) mediated protoplast transformation, electroporation and microprojectile bombardment. Among these methods, *Agrobacterium tumfaciens* mediated transformation is the most common, used in all of the fruit tree-transformation. It is demonstrated that competent cells for transformation are located in the newly formed callus growing from the cambial ring. Conditions conducive to further development of this callus, such as treatment of explants in the medium rich in auxins, resulted in more pronounced formation of cambial callus and slower shoot regeneration process, both in *Agrobacterium*-inoculated and non-inoculated explants. Furthermore,

co-cultivation in the medium rich in auxins caused a significant increase in the rate of actively dividing cells in S-phase when cells are more prone to integrate foreign DNA. Factors affecting *Agrobacterium*-mediated transformation efficiency included mode of pre-cultivation, temperature of co-cultivation and presence of acetosyringone (AS); Increasing the wounded area of explants by cutting the internodes longitudinally into two halves, and optimization of inoculation density, dramatically enhanced both regeneration and transformation frequency. Adding 2,4-dichlorophenoxyacetic acid (2,4-D) in the explant pretreatment medium and the co-culture medium improved the transformation efficiency in citrus.

To improve the efficiency, Escobar (Escobar et al. 2000) used the green fluorescent protein gene (*gfp*) as a scorable marker in walnut somatic embryo transformation. Their work suggested that the use of *gfp* as a selectable marker could significantly reduce labor, cost, and time in the walnut somatic embryogenesis-based transformation system.

The work on papaya indicated that gene dosage and the development stage of plant would also affect the resistance of cultivars 'Sun Up' and "Rainbow". Young and older hemizygous 'Rainbow' plants were resistant to the homologous PRSV (papaya ring spot virus) HA (99.8% homology to CP transgene), while only older 'Rainbow' plants were resistant to the other Hawaiian isolates (96.7% homology) (Tennant et al. 2001). It was concluded that the position of the heterologous sequences in the recombinants influenced their pathogenicity in 'Rainbow'.

Silencing of the transgene is a problem occurred in the transformation, especially in the perennial trees. Mexican lime (*Citrus aurantifolia*) plants transformed with the p25 coat protein gene of citrus tristeza virus (CTV) presented more than 30% silence among the regenerates under non-selective conditions; and in all cases, silencing affected the transgenes incorporated; inverted repeats as well as direct repeats and even single integrations could also trigger gene silencing (Dominguez et al. 2002). Among the grapefruit (*Citrus paradisi* Macf. cv Duncan) plants transformed with several sequences from CTV, a great variability in titer was observed in both controls and transgenic plants, and all were apparently susceptible to the virus, although some transgenic plants averaged lower titers of virus than controls. However, the transgenic plum clone C-5 carrying the PPV-coat protein inserts had been proved as the most stable high-level resistance to plum pox virus (PPV) after four years of testing in the greenhouse.

3.3 Target Genes Introduced into Fruit Trees

Different traits have been modified in transgenic fruit trees, which comprise altered processing and storage qualities, resistance to abiotic stresses, insects, viruses and bacteria, improvement of rooting and shortening the juvenile phase.

3.3.1 Abiotic-stress Tolerance

Tolerance or resistance to stresses such as drought and salinity is one of the main goals of fruit breeding. There are several successful reports of fruit trees with modified stress tolerance by genetic transformation such as drought tolerance of Carrizo citrange, salt tolerance of apple, persimmon, Carrizo citrange etc.

3.3.2 Shortening of the Juvenile Phase

The flower regulating genes like *LEAFY* (LFY) and *APETALA1* (AP1) from *Arabidopsis* were used to shorten the juvenility. Pena et al. (2001) demonstrated that the permanent expression of these two genes under the control of the commonly used cauliflower mosaic virus 35S promoter significantly shortened the juvenile phase of citrus trees. Plants harboring one of the two genes flowered and produced fruit within one year after the seed germination. The accelerated juvenile period was heritable in crosses with non-transformed plants. Recently, the transgenic apples expressing antisense MdTFL gene (homologous to *Arabidopsis TERMINAL FLOWERING1* gene from apple) were reported flowering at about 8 to 15 months after grafting onto rootstocks, while the non-transformed plants did not flower in 5 years. And most recently, Endo et al. (2005) reported that they obtained transgenic trifoliate orange (*Poncirus trifoliate* L. Raf.) showed very early flowering and fruiting (i.e. 12 weeks after transfer to the greenhouse) when overexpressed *Citrus* FT (CiFT, homolog of *Arabidopsis FLOWERING LOCUS T*).

3.3.3 Disease Resistance

Viruses can cause severe losses by substantially reducing yield, affecting fruit quality, and shortening the lifespan of infected plants. Genetic transformation allows the insertion of specific virus resistance traits directly into desirable elite varieties (see Chap. 9). Besides viruses, fruit genetic transformation such as endochitinase or exochitinase, attacin E, polygalacturonase inhibiting protein (PGIP) and pathogenesis-related proteins like glucanases and chitinases were also reported.

3.3.4 Insect Resistance

As well as diseases harming fruit trees, insects often destroy the fruit tree on leaf, stem, root, flower and fruit, and lead to the decrease of both quality and production. Genes like avidin or strepavidin, cowpea trypsin inhibitor (CpTI) or *Bacillus thuringiensis* toxin (see Chap. 12) for insect control have been introduced into apple, walnut and persimmon, respectively.

3.3.5 Rootstock Improvement

Poor rooting ability is one of the major problems for cultivation of fruit trees both in vitro and in vivo. Dwarfness is another target for improvement of rootstocks (Chap. 16). *Rol* A, B, C genes originated from *Agrobacterium rhizogenes* single or combined together could resolve these problems partially. Genetic transformation thus provides a prospect and have been used widely for several fruit tree species including: apple, chestnut, kiwifruit, olive, pear and persimmon.

3.3.6 Fruit Improvement

Most fruit trees are cultivated for fresh fruit; thus, improvement of fruit quality or storage can be very attractive for consumers. Genetically modified fruit trees for such a target have been reported in several fruit tree species like citrus, apple, chestnut and passion fruit. Genes modified fruit traits include polyphenol oxidase (PPO), carotenoid biosynthetic genes controlling fruit and juice color, pTA29-barnase gene for seedless, 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) or synthesis (ACS) controlling fruit ripening, the juice quality related pectin methylesterase gene.

3.4 Progress in Genetic Transformation of Fruit Trees

In the past 15 years, significant progress in recovering transgenic fruit trees was obtained, and some details are given below.

3.4.1 Apple

Apple (*Malus pumila* Mill.) is one of the most common fruit trees and has attracted many researchers in genetic transformation. Since the first transgenic apple was reported in 1989 (James et al. 1989), more than 20 cultivars have been transformed successfully (Table 3.1). In most cases, *Agrobacterium tumefaciens*-mediated leaf disc transformation were adopted. Protoplast-mediated transformation seemed not to be available, since no integrated transgenic apple plant was regenerated with this method. Among these transformation cases, five agronomic traits were targeted: (1) genes including scab resistant genes like wheat puroindoline B (pinB), homologues of *Cladosporium fulvum* resistance genes of Vf (HcrVf2) from wild *Malus* species, endochitinase or exochitinase of the biocontrol fungus *Trichoderma atroviride*; (2) fire blight resistant genes like antimicrobial peptides (AMPs), endogenous alleles of the S-gene, an inducible antibacterial

protein from *Hyalophora cecropia pupae* (attacin E), a modified cecropin SB37 gene (MB39); (3) rooting ability increasing genes like *rol* A, B, C; (4) insect resistant genes like the biotin-binding proteins avidin or strepavidin; and (5) fruit traits modifying genes like PPO (polyphenol oxidase), ACO and ACS (Table 3.1).

The most destructive disease of commercial apple orchards is scab caused by *Venturia inaequalis*, which attacks both the foliage and the fruit, thus resulting in reduced yield. Several apple cultivars highly susceptible to this disease such as 'Gala', 'MacIntosh', 'Galaxy', and the hybrid 'Ariane' had been the subject of attempts to improve scab resistance by genetic engineering. Therefore, transgenic plant production represents the basis for further investigation of resistance mechanism and a step toward gene therapy of scab-susceptible cultivars that currently dominate the apple industry. As far as rootstock breeding is concerned, *Agrobacterium rhizogenes* and its root loci of *rol* A, B, C are considered helpful and can be transferred into plants. Several apple rootstocks such as M_{26}, A_2, $Jork_9$, M_7 and Marubakaidou had been transformed successfully, which helped to resolve the difficulty of rooting and thus be attractive both for increasing productivity and for dwarfness. The antisense PPO gene was introduced into apple callus and shoot to reduce browning, which was not only useful for the food industry but also for the studies of the metabolism of polyphenols and the function of PPO (Murata et al. 2001). Transgenic 'Greensleeves' apple with reduced ethylene synthesis was obtained by expressing antisense apple ACO or ACS, which resulted in improved firmness and low ester accumulation in fruit for elongating the storage and shelf life (Dandekar et al. 2004).

3.4.2 Apricot

Transformation of apricot (*Prunus* L.) was first reported in 1992 (da Camara Machado et al. 1992) and a successful integration of a viral coat gene of Plum Pox Virus (PPV) into *Prunus armeniaca* was obtained (see Chap. 9). Transformation in almond cultivars like 'Supernova', paper shell almond (*Prunus dulcis* Mill.) cultivars 'Nonpareil' and 'Ne Plus Ultra' were realized.

3.4.3 Cherry

In cherry (*Cerasus avium* L.) breeding, the upmost task is to reduce the large size of the tree canopy. Thus the ideal cherry rootstock should be able to reduce the size of the scion, be easy to root, and be resistant to biotic and abiotic soil stresses. Thus, transgenic rootstocks Colt (*Prunus avium* × *P. pseudocerasus*) and Mazzard F12/1 (*P. avium* L.) with increased rooting ability were produced after inoculation with *Agrobacterium rhizogenes* as reported by Gutierrez-Pesce et al. (1998).

3.4.4 Chestnut

Transformation in chestnut (*Castanea Mill*) was performed either by *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*-mediated transformation with different kinds of explants including androgenic embryos, hypocotyls, globular or early-torpedo stages and stem segments. Chestnut plants had been drastically reduced as a result of ink disease and blight disease caused by fungi, and therefore genetic transformation for resistance to fungal diseases was most important in chestnut breeding. A typical transformation research of producing rootstocks with higher levels of tolerance to fungal disease was reported (da Costa Seabra and Pais 1999), in which the β-1,3-glucanase (*glu*) and chitinase (*chi*) gene were introduced into European chestnut (*Castanea sativa* Mill).

3.4.5 Citrus

Citrus (*Citrus* L.) is the most extensively grown fruit crop worldwide. And there are many in vitro protocols such as callus and cell suspension cultures, organogenesis induction, and protoplast isolation that are viable for genetic transformation. Transgenic citrus plants have been obtained by direct DNA transfer into protoplasts, co-cultivation of internodes or epicotyl segments with *Agrobacterium* and particle bombardment of nucellar embryogenic cell suspensions. The most widely used method of gene transfer in citrus is the *Agrobacterium*-mediated transformation of epicotyl segments. Using this system, transgenic plants of citrus species and relatives were obtained, including sweet oranges, sour oranges, limes, grapefruit, and Carrizo citrange rootstock. As for the transgenes, more and more agronomic genes were introduced into citrus plants. These include genes encoding the citrus tristeza virus (CTV) coat protein from different citrus tristeza virus strains (Chap. 9), citrus mosaic virus (CiMV) coat protein, the halotolerance gene HAL2 originally isolated from yeast that confers tolerance to salinity, Arabidopsis *LEAFY* and *APETALA1* genes and citrus FT gene that promote early flower initiation, carotenoid biosynthetic genes from fruits of *C. paradisi* cv. Flame that control fruit and juice color, and *CS-ACS1* gene from *Citrus sinensis* that controls the ethylene biosynthesis, Xa21 providing broad spectrum *Xanthomonas* resistance in rice with potential citrus canker disease resistance. And recently, chimaeric ribonuclease gene (barnase) for seedlessness and the juice quality related pectin methylesterase gene (PME) from Valencia orange was introduced into sweet orange by Li et al. (2003) and Guo et al. (2005) respectively.

Among the transformation methods, there are three worthy of note. First, mature tissue transformation. The reliable method for the production of mature transgenic citrus plants via *Agrobacterium* was proceeded with *C. sinensis L.* Osbeck cultivars ‘Pineapple’, ‘Hamlin’, ‘Pera’, ‘Valencia’ and ‘Natal’,

though only the former two cultivars succeeded. Second, thin epicotyl sections transformation for Carrizo citrange (*C.* × *Poncirus trifoliata*), and Swingle citrumelo (*C. paradisi* × *P. trifoliata*) was provided. Third, a further increase in the genetic transformation efficiency in citrus had been obtained by favoring the contact of bacteria and the cambial region. The use of longitudinally cut internodes or epicotyls to expose the cambial region could improve the transformation rate.

3.4.6 Grapevine

Since the first transgenic grape (*Vitis vinifera* L.) plant was reported in 1990 (Mullins et al. 1990), transgenic grape could be generated by transformation of embryogenic calli and somatic embryos. And using leaves as explants to obtain transgenic grape via somatic embryogenesis in *Vitis vinifera*, and shoot and plantlet as the explants for transformation in hybrid 'Gravesac' have been proved successful.

The most significant progress in grape genetic engineering was the obtaining of transgenic grape cultivars resistant to two damaging viruses, i.e. grapevine fan leaf virus (GFLV) and grapevine chrome mosaic virus (GCMV). Transgenic plants were tested under field conditions. Further information about virus resistance can be found in the "virus resistance" chapter. Other agronomic genes introduced into grape plants were the rice chitinase gene (RCC2) against powdery mildew and the eutypine detoxifying gene (Vr-ERE) encoding an NADPH-dependent aldehyde reductase gene to increase detoxification capacity.

3.4.7 Kiwifruit

Kiwifruit (*Actinidia deliciosa*) is a recent introduction among the promising fruit crops. Transformation in kiwifruit first reported in 1991 (Uematsu et al. 1991) includes *Agrobecterium rhizogenes*, *Agrobacterium tumefaciens* and PEG-mediated transformation. Agronomic genes were introduced into kiwifruit plants such as rolABC genes to promote rooting, a soybean beta-1, 3-endoglucanase cDNA to enhance resistance to *Botrytis cinerea*, a rice *Oryza sativa* homeobox 1 (OSH1) to study the mechanisms of morphogenesis (Kusaba et al. 1995) and a stilbene synthase gene from three *Vitis* spp. (*V. vinifera*, *V. labrusca* and *V. riparia*) (Kobayashi et al. 2000) to provide potential beneficial effects on health.

3.4.8 Papaya

Transformation in papaya (*Carica papaya*) was mainly dependent on *Agrobacterium tumefaciens* mediated method; in addition, transformation by

Agrobacterium rhizogenes-mediated and particle bombardment was also successful. Embryogenic tissues including callus, somatic embryos and zygotic embryos were considered to be the best explants and were used widely for transformation because of their high potential of regeneration; additionally, leaf discs and petioles were used in some transgenic experiments.

Transgenic technology applied in papaya majored on virus resistant breeding, especially for PRSV (papaya ring spot virus; for details see Chap. 9). A recent report elucidated that a stilbene synthase gene (Vst1) from grapevine (*Vitis vinifera* L.) had been introduced into papaya, which resulted in increasing resistance to the pathogen *Phytophthora palmivora*. This provided a new way to control fungal diseases in papaya breeding, since papaya is susceptible to a variety of fungal diseases, including root, stem, and fruit rot caused by the pathogen *Phytophthora palmivora*.

3.4.9 Peach

The development of transformation in peach (*Prunus* L.) lags behind other fruit trees like citrus and apple because of the difficulty in its tissue culture and regeneration in vitro. The first transformation event in peach was reported by Smigocki (Smigocki et al. 1987). *Agrobacterium*-mediated transformation and microprojectile bombardment were the main transformation methods, and the explants were leaf, stem segments, embryogenic cells, immature embryos and embryo sections from mature seeds. Most of them were reported as the development of transformation system, few addressed the agronomical traits.

3.4.10 Pear

Since the first transgenic pear (*Pyrus* L.) plant was obtained in 1996 (Mourgues et al. 1996), a significant progress in pear resistance breeding via genetic transformation was achieved. There were several reports on successful transformation with different desirable genes such as an iron chelator protein encoded by the bovine lactoferrin gene, attacin E (anti-bacterial genes SB-37), Shiva-1 and lysozyme isolated from the T4 bacteriophage. All cases were performed by *Agrobacterium tumefaciens*-mediated transformation with leaf or excised cotyledons as the explants. To get high efficiency transformation of pear, several efficient and repeatable methods have been established; new method was established for the transformation of two recalcitrant cultivars ‘La France’ and ‘Silver’ by using axillary shoot meristems as explants. To improve rooting ability, the *rol* B gene was introduced into pear rootstock BP10030 (*Pyrus communis*). By introducing pear (cv. ‘La France’) antisense cDNAs encoding ACC synthase (LF-ACS) and ACC oxidase (LF-ACO) into pear, ethylene synthesis was reduced, thus better firmness in pear fruit and elongation in shelf life were realized.

3.4.11 Persimmon

The first transgenic persimmon (*Diospyros kaki* L.) was reported by Tao (Tao et al. 1994). Transformation in persimmon depended on *Agrobacterium tumefaciens* mediated transformation with explants such as leaf-disc, callus and hypocotyl segments. The first report of tolerance to salt in an agriculturally important species of woody plants came from persimmon transformed with the codA (a gene encoding choline oxidase) of *Arthrobacter globiformis*. Later, an apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH) and a cDNA encoding sorbitol-6-phosphate dehydrogenase (S6PDH) were transferred into persimmon respectively to enhance salt stress tolerance. Other genes were introduced into persimmon cultivars including the rolC gene from *Agrobacterium rhizogenes*, the PGIP gene (Tamura et al. 2004) from pear encoding the fruit (*Pyrus communis*) polygalacturonase inhibiting protein and the cryIA(c) encoding the insecticidal crystal protein fragment of *Bacillus thuringiensis* (Bt).

3.4.12 Plum

Mante et al. (1991) established the first transformation system of plum (*Prunus domestica* L.). Plum pox disease or sharka is of economic importance, affecting yield and vitality of infected stone fruit trees including peach, plum, apricot and cherry. Genetic transformation was first used in 1994 (Scorza et al. 1994) to fight against sharka virus and a new Plum cultivar resistant to PPV was developed. Transgenic plums were obtained by *Agrobacterium tumefaciens* with either hypocotyl slices or embryogenic callus as transformation explants. Till now, transgenic plum clone C5 was considered to be highly resistant and stable after several years of testing both in the greenhouse and under field conditions.

3.4.13 Walnut

The earliest transformation of walnut (*Juglans* L.) was performed in 1988 (McGranahan et al. 1988) by *Agrobacterium*-mediated transformation. Somatic embryos were the main explants used for the transformation. Genes were introduced into walnuts including a walnut antisense chalcone synthase (chs) gene to improve flavonoid content and rooting ability and the Bt cryIA(c) gene to protect against insects. To improve rooting ability, the rolABC genes were introduced into walnut by *Agrobacterium*-mediated transformation.

3.4.14 Others

Mango (*Mangifera indica* L.) occupied an important place in agriculture with large numbers of fresh fruit produced every year. Transformation in mango

was concentrated on establishment of transformation systems with reporter genes; there is no advanced report on introducing agronomically important genes into mango.

In order to improve fruit quality and shelf life of the yellow passion fruit (*Passiflora coerulea* L.), experiments were carried out on the introduction of an antisense melon ACC oxidase gene (CMe-ACO1) into leaf explants via *Agrobacterium tumefaciens*, followed by plantlet regeneration.

Avocado (*Persea* Mill.) cultivar 'Thomas' was transformed by *Agrobacterium tumefaciens* strain A208 with the embryogenic cultures as the explants. However, only somatic embryos containing the reporter gene was obtained.

3.5 Conclusions

Since the first report of the transgenic fruit in the late of 1980s, great progress has been achieved; this includes the transformation system establishment in most fruits, and the successive regeneration of genetic transformed papaya with resistance to papaya ring-spot virus (Fitch et al. 1990), the early flower citrus (Pena et al. 2001), etc. However, the present impact of the genetic transformation of fruits to the industry is still limited, compared with crops such as soybean. The main reasons for this come from the following aspects. First, most fruits are perennial crops with a long juvenility and difficulty for in vitro regeneration. This characteristic leads to longer research periods to get the desired trait, especially fruit traits. Second, the research background including the cloning of gene, and the genetic map etc. is very poor for most fruit crops. The weak background impacted transformation research, and most of the work was based on the experience or skill of the researchers. One's work could not easily begin on the basis of others. Third, the public concern for GMO safety, especially regarding the genomically modified freshly eaten fruits to some degree, limits the research of transgenic fruit crops. With more understanding of, and improvement in, transgenic technology, it is believed that more and more transgenic fruits with modified traits will be created in the future, and it will be an important alternative channel for fruit breeding.

Based on the present situation, it can be predicted that improvement of tolerance to biotic and abiotic stresses via genetic transformation will resulted in the best possible results for all traits and will be utilized in the near future. And improvement on the rootstock with genetic transformation will have more advantages than on the scion, since the rootstock plays a very important role in tree tolerance to stresses, and less concern regarding GMO safety is attracted.

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4 Genetic Transformation of Some Tropical Trees, Shrubs, and Tree-like Plants

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4.1 Introduction

Trees play a vital role at the ecological, social and economical levels. Trees contribute greatly to maintenance of biodiversity, ecosystem and global climatic changes. They help in soil retention, carbon budgeting, and provide mankind with a wide range of products including fruits, nuts, oil, spices, wood, pulp, wood products, latex and secondary metabolites. Depending upon their geographical distribution, trees are grouped as tropical, sub-tropical, temperate and sub-temperate. The classical methods of tree improvement are based on selection and breeding. Biotechnology has tremendous potential in tree improvement. Genetic transformation not only complements conventional selection and breeding of superior trees but also makes a major contribution to overcome constraints like long breeding cycles, species barriers and narrow genetic pools in cultivated commercial tree crops. The first successful transformation in trees was achieved in 1987 by Fillatti et al. Since then progress has been slow but steady and has witnessed many new inventions and techniques over past decade, which have been reviewed extensively (Merkle and Dean 2000; Pena and Seguin 2001; Herschbach and Kopriva 2002; Diouf 2003; Gallardo et al. 2003; Gartland et al. 2003). The main theme to attempt genetic transformation in trees is the improvement of productivity and quality. The potential of production of trees with novel traits is one of the most distinct benefits of genetic transformation. The idea of using rubber and banana for molecular farming of desired products is also gaining momentum.

This chapter reviews the genetic transformation studies carried out in some of the economically important tropical trees and tree-like plants, mainly banana, cocoa, coffee, eucalyptus, oil palm and rubber trees which are considered as cash crops and are the major source of income in the tropics. Work done on some temperate species of eucalyptus have also been considered in this review. In view of the substantial work done on genetic transformation in these species, an attempt has been made to compile the scattered information and present it on a single platform. The review is based on the

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extensive literature survey from all available sources. Detailed information on the transformation studies carried out in the above-mentioned species with respect to tissue culture system used, genes transferred and mode of transformation is summarized in Table 4.1.

4.2 Genetic Transformation Studies

4.2.1 Banana, *Musa* sp.

Banana is a member of the genus *Musa*, belonging to family *Musaceae*. This is one of the earliest crops, cultivated since 4000 years (Purseglove 1975; Ganapathi et al. 2003). Banana (*Musa* sp.) is the fourth most important global food crop after rice, wheat and maize in terms of gross value of production (Moffat 1999). The East and Central Africa sub-region alone produces nearly 20 million tons of banana annually (Tripathi et al. 2004).

Banana fruit is used as a staple food owing to its good nutritive value with high carbohydrates 22.2%, fiber 0.84%, protein 1.1%, fat 0.2% and water 75.7% (Ganapathi et al. 2003).

Banana is known for its high susceptibility to various fungal pathogens, viruses, insects and nematodes which ultimately affect the fruit yield and quality (Sagi et al. 1998). Among the fungal diseases, panama wilt and sigatoka disease are of major concern. The average annual loss of yield due to nematode diseases is estimated to about 20% worldwide (Sasser and Freckman 1987) and about 40% or greater in the areas prone to tropical storms. The most widely damaging nematode is *Radopholus similes* (burrowing nematode). Nematode management in banana is mainly based on crop rotation and chemical control (Gowen and Quénéhervé 1990). To date, chemical control is the only viable and affordable way to control nematode attack. Commonly used nematicides are organophosphates and carbamates (Whitehead 1998), which have adverse effects on the environment and the health of farmers. The major diseases of banana and their causative pests and pathogens are listed in Table 4.2.

Conventional breeding of banana has been impaired due to its triploid nature (Ganapathi et al. 2001), production of pollen by only few diploid clones, and male/female sterility (Novak et al. 1989). Transformation studies in banana are being carried out (i) to confer resistance towards nematodes, fungal pathogens and viruses, (ii) to improve fruit quality and its shelf life, and (iii) to produce pharmaceutically important peptides in fruit, i.e., edible vaccine.

Transient *GUS* expression in banana was reported for the first time by Sagi et al. (1994) by electroporation of protoplasts. Gene transfer using particle bombardment was reported by Sagi et al. (1995), Dugdale et al. (1998), Schenk et al. (1999), and Becker et al. (2000). Most of the experiments used

Table 4.1. Genetic transformation studies on banana, cocoa, coffee, eucalyptus, oil palm and rubber tree

Plant species	Tissue/Culture System Used for transformation	Genes transferred & promoters used (P)	Mode of Transformation	Reference
Banana				
Musa cv. Bluggoe (ABB)[a]	Protoplasts isolated from ECS	*GUS* (P-*uid A*)	Electroporation	Sagi et al. (1994)
Musa cv. Grand Nain	Meristems and corm slices	*NPTII, uidA* (P-rice *actin)*	*A. tumefacians*	May et al. (1995)
Musa sp.[c]	ECS	*GUS, HPT*	Biolistic	Sagi et al. (1995)
Musa sp.	NA	NA	Biolistic	Dugdale et al. (1998)
Musa sp.	NA	*ACCS*	NA	Liu et al. (1999)
Musa sp.	NA	cDNAs encoding three different defensins	NA	Remy et al. (1998)
Musa sp.	ECS	NA	Biolistic	Schenk et al. (1999)
Musa sp. Cavendish banana cv. Grand Nain	ECS	*NPTII, uidA* (P-BBTV, *CaMV35S*, MP)	Biolistic	Becker et al. (2000)
Musa cv. Rasthali (AAB)	ECS	Antimicrobial peptide -MSI-99	*A. tumefaciens*	Chakrabarti et al. (2000)
Cavendish banana (AAA)[c]	NA	Rice cystatin (*OcIΔD86*)	*A. tumefaciens*	Engler et al. (2000)
Musa cv. Rasthali (AAB)	ECS derived from shoot apex	*GUS, als* (P-*Gelvin*, *ubi1)*	*A. tumefacians*	Ganapathi et al. (2001)

(*continued*)

Table 4.1. (*continued*)

Plant species	Tissue/Culture System Used for transformation	Genes transferred & promoters used (P)	Mode of Transformation	Refeencee
Musa cv. Grand Nain (AAB) & Lady Finger (AAB)	ECS derived from male flower inflorescence	*HPT, GUS, NPTII,* modified *GFP*	*A. tumefaciens* (AGL1, LBA4404)	Khanna et al. (2004)
Banana and plantain	ECS	Five AMP	Biolistic	Tripathi et al. (2004)
Cocoa				
Theobroma cacao[c]	Callus	NA	NA	Sain et al. (1994)
T. cacao	Somatic embryos	*Chitinase*, *GFP*	*A. tumefaciens*	Maximova et al. (2003); de Mayolo (2003)
Coffee				
C. arabica	Protoplast	CAT, KAN	Electroporation pGA472	Barton et al. (1991)
C. arabica cv. Catturra	Protoplast	*GUS, NPTII*	*A. tumefaciens* PGV226035GUS-INT	Spiral and Petiard (1991)
C. arabica cv. Caturra	Hypocotyls of *in vitro* germinated seeds	*AGS*	*A. tumefaciens* 17613; 3565; 3569; 391; A281 (pTi Bo542)	Ocampo and Manzanera (1991)
C. arabica	NA	*GUS, NPTII*	*A. tumefaciens*; C58 (pGV2260); C58 (pMP90); AGL1 (pTiBo542)	Gr'ezes et al. (1993)
C. canephora cv. Robusta	NA	*NPTII, GUS*	*A. tumefaciens*	Spiral et al. (1993)
C. arabica & C. canephora	Somatic embryos	*GUS, NPTII*	*A. rhizogenes*	Spiral & Petiard (1993, 1999)
C. arabica	NA	*KAN, GUS*	*A. tumefaciens*	Gimenez et al. (1994)
C. arabica	Protoplast	*GUS*	Electroporation pCH1	Van Boxtel (1994)

C. arabica cv. Colombia	NA	*NPTII* , *GUS*	PEG	De Pena (1995)
C. arabica	Microcuttings; suspension cultures; somatic embryos	GUS	Biolistic pCH1; pUBQ1; pBMCV; pP1GK	Van Boxtel et al. (1995)
C. canephora	NA	Chlorsulfuron resistance gene, *GUS, NPTII*	*A. tumefaciens* (disarmed)	Spiral and Petiard (1999)
C. arabica	Embryogenic calli	*GUS, HPT, NPTII* (EHA101)pIG121-Hm	*A. tumefaciens*	Hatanaka et al. (1999)
C. arabica and *C. canephora*	Somatic embryos	*cry1Ac* from *Bt*	*A. tumefaciens* (LBA4404)	Leroy et al. (2000, 2001)
C. arabica	Single embryogenic cell line derived from a leaf section	dsRNA of *CaMXMT1*	*A. tumefaciens*	Ogita et al. (2004)
C. canephora[c]	Somatic embryos	dsRNA of *CaMXMT1*	*A. tumefaciens*	Ogita et al. (2004)
Eucalyptus				
Eucalyptus gunnii[a]	Protoplasts	NA	Electroporation using PEG	Teulieres et al. (1991)
E. citridora[a]	Protoplasts	NA	Electroporation using PEG	Manders et al. (1992)
E. globulus	Six day old embryos	GUS	Biolistic	Rochange et al. (1995)
E. globulus	Zygotic embryos	GUS	Biolistic	Serrano et al. (1996)
E. grandis × *E. urophylla*	NA	NA	*A. tumefaciens* (12 wild types) and *A. rhizogenes* (five strains)	Machado et al. (1997)
E camaldulensis[c]	Leaf explants	*NPTII, GUS*	*A. tumefaciens* (A6, LBA4404, GV3111, AGLI and GV3850)	Mullinis et al. (1997)

(*continued*)

Table 4.1. (*continued*)

Plant species	Tissue/Culture System Used for transformation	Genes transferred & promoters used (P)	Mode of Transformation	Reference
E camaldulensis[c]	Leaf explants	*NPTII, GUS*	*A. tumefaciens*	Ho et al. (1998)
E. globulus	NA	NA	*A. tumefaciens*	Moralejo et al. (1998); Bandopadhyay et al. (1999)
E. nitens	NA	NA	NA	Bandopadhyay et al. (1999)
E. grandis[c]	NA	Glyfosate resistance gene	*NA*	Llewellyn (1999)
E. camaldulensis[c]	NA	*Cry3A* from *Bt*, *BAR*	*NA*	Harcourt et al. (2000)
E. camaldulensis[b]	Mature tissues	*C4H* from *Populus tremuloides*	*A. tumefaciens* (CIB542) pSC4H and pASc4H	Chen et al. (2001)
E. grandis	Seeds germinated for 2 days and 15 day old seedlings	*GUS*	*A. tumefaciens* (sonication for 30 s prior to cocultivation)	Gonzalez et al. (2002)
E. camaldulensis[c]	NA	Antisense *Ntlim1, KAN*	NA	Kawaoka et al. (2003)
E. camaldulensis	NA	CAD antisense full-length cDNAs from *E. gunnii* or *N. tabacum*	*A. tumefaciens*	Valério et al. (2003)
E. globulus	Apical stem segments	*GUS*	*A. tumefacians* (AGL1, LBA4404)	Antanas et al. (2005)

Oil palm				
E. guineensis	Callus	Antisense *D9SAD*	NA	Abdullah et al. (1997)
Elaeis guineensis	Embrygenic calli	*GUS*	*NA*	Parveez et al. (1997, 1998)
E. guineensis	Immature embryos (11±12 weeks after anthesis)	*GUS, HPT*	*A. tumefaciens* with sonication Pcambia1301	Roberts et al. (1997)
E. guineensis	NA	*CpTI*	NA	Abdullah (2005)
Rubber tree				
Hevea brasiliensis	In vitro and in vivo seedlings	NA	*A. tumefaciens* (541)	Arokiaraj et al. (1991)
H. brasiliensis	Callus	*GUS, NPTII, CAT*	Biolistic	Arokiaraj et al. (1994)
H. brasiliensis	NA	*GUS, NPTII, KAN*	*A. tumefaciens* (GV2260) p35SGUSINT	Arokiaraj et al. (1998)
H. brasiliensis	NA	Gene for HSA	NA	Arokiaraj et al. (2002)
H. brasiliensis[c]	NA	A mouse antibody fragment (single chain variable fragment (scFV) gene, *GUS*	*NA*	Yeang et al. (2002)
H. brasiliensis[c]	Immature anther calli (aged two months)	*HbSOD, uidA, NPTII* (P-*CaMV35S)*	*A. tumefaciens*, pDU96.2144	Jayashree et al. (2003)

[a]Transient expression
[b]Stable expression
[c]Plants grown in greenhouse/field planting
GUS-ß glucuronidase; *NPTII*-Neomycin phosphotransferase II; *AGS*-Agropine synthase; *GFP*-Green Fluorescent protein; *CAT*-cholorompheniсol acetyl transferase; *HPT*-Hygromycin phosphotransferase; *Als*-confers resistance to sulfonyl urea herbicide; *KAN*-Kanamycin resistance gene; *BAR*-Basta (herbicide) resistance gene; ECS-embryogenic cell suspension; *Bt-Bacillus thuringienesis*; cpTI-cowpea trypsin inhibitor; P-Promoter used; MP-maize polyubiquitin; HSA-Human serum albumin; *CaMXMT1*-gene involved in caffeine biosynthesis; NA-not available

Table 4.2. Major diseases and its causative organisms in banana

Disease	Causative organism
Nematode	
Nematode diseases	*Radopholus similes, Pratylenchus goodeyi, Pratylenchus coffeae, Helicotylenchus multicinctus, Meloidogyne sp.*
Fungal	
Black Sigatoka	*Mycosphaerella fijiensis*
Yellow Sigatoka	*Mycosphaerella musicola*
Septoria leaf spot	*Mycosphaerella eumusae*
Panama wilt	*Fusarium oxysporum*
Moko disease	*Pseudomonas solanacearum*
Leaf spot	*Cercospora musae*
Anthracnose	*Gloeosporium musarum*
Viral	
Bunchy top	Banana Bunchy Top Virus (BBTV)
Bract mosaic	Banana Bract Mosaic Virus (BBMV)
Infectious chlorosis	Cucumber Mosaic virus

Source: Ganapathi et al. (2003); Bridge et al. (1997); Carlier et al. (2000)

embryogenic cell suspensions. *Agrobacterium* mediated transformation of embryogenic cell suspension was carried out by Ganapathi et al. (2001) and transgene expression was confirmed in regenerated transgenic plants. Other tissues like shoot tips (May et al. 1995; Tripathi et al. 2002), apical meristems, corm slices (May et al. 1995) have also been used.

Transformation studies towards conferring disease resistance is of great significance in banana as so far there is no natural resistance observed towards major pests and diseases in any of the banana cultivars. However, partial nematode tolerance and resistant germplasm have been identified in *Musa* genepool (Pinochet 1996). Studies on screening of the natural genepool of banana to identify nematode-resistant trait will be helpful in conferring nematode resistance. Introduction of proteinase inhibitor genes to sensitive plants provides an attractive tool for introducing nematode resistant trait (Ryan 1990). Many nematodes have cysteine proteinases as digestive enzyme (Atkinson 2000). Cysteine proteinase inhibitors (cystatins) found in seeds of sunflower, cowpea, soybean, maize and rice blocks the activity of cysteine proteinases (Atkinson et al. 1995). Using this strategy, nematode tolerant transgenic plants have been produced in a number of plant species (Urwin et al. 1995, 1997, 2000, 2001, 2003; Vain et al. 1998) and a way has also been explored to introduce nematode resistance in banana. Engler et al. (2000) transferred rice cystatin (*OcIΔD86*) gene under control of three different promoters to Cavendish banana (AAA) via *Agrobacterium tumefaciens* and

generated transgenic plants. Atkinson et al. (2004) continued further analysis of the expression level of *OcIΔD86* gene and resistance acquired by these transgenic plants. Of the three promoters tested, ubiquitin promoter (*UBI-1*) showed the maximum expression and transgenic plants acquired resistance up to 70±10%.

Antimicrobial peptide-MSI-99, an analog of megainin-2, a 23 amino acid long peptide isolated from the African clawed frog *Xenopus lavies* (Zasloff 1987), has been transferred to Banana cv. Rasthali to obtain resistance against fusarium wilt (Chakrabarti et al. 2003). The transgenic banana plants thus obtained were screened for resistance to fusarium wilt. Five plants (transformed with pMSI164) and 11 plants (transformed with pMSI168) showed resistance to *F. oxysporum*. Molecular and biochemical analysis confirmed the presence of introduced genes in various transgenic lines. Tripathi et al. (2003) demonstrated the expression of AMP gene in the fruit, creating the opportunity to develop resistance against pre- and post-harvest diseases such as cigar-end rot and crown rot. Recently it has been suggested that the antimicrobial peptides can also be used to provide resistance against bacterial pathogens (Tripathi et al. 2004).

Banana Bunchy Top Virus (BBTV) is responsible for major crop loss in banana. So far no natural resistance against BBTV has been identified in any of the *Musa* sp. Interference with the normal replication, encapsidation or movement of the virus in the plant, expression of heterologous antiviral proteins inhibiting viral replication or translation are the different strategies used to develop BBTV resistance (Sagi 2000). Studies on the BBTV genome revealed that the genome is composed of six circular ssDNA in sense (Harding et al. 1993; Burns et al. 1995). Further studies related to promoter activity were carried out by Dugdale et al. (1998). This strategy can be exploited for developing resistance against banana streak virus.

Increasing the shelf life of banana fruit and production of vaccine in the fruit are the two major areas of commercial potential. Extensive research is being done on these lines and initial results are promising. Banana is a tropical fruit and is very susceptible to over-ripening triggered by ethylene. The ethylene biosynthetic pathway described for ripening of apples (Adams and Yang 1979) is also observed in bananas (Hoffman and Yang 1980). Unripe banana show a constant but low level of ethylene production until the onset of ripening. ACC synthase (ACCS) and ACC oxidase (ACCO) are two important enzymes involved in the ethylene biosynthesis. The mechanism of ethylene biosynthesis and triggering of ripening is now well understood (Dominiguez and Vendrell 1993; Oetiker and Yang 1995; Clandennen et al. 1997). A number of reports are available on cloning of ACCS gene in different plants like tomato (van der Straten et al. 1990), apple (Dong et al. 1991), passion fruit (Mita et al. 1998), cucumber (Shiomi et al. 1998), melon (Yamamoto et al. 1995), and others. ACCS gene has also been cloned from banana (Liu et al. 1999). The information generated through these studies can be utilized for increasing the shelf life of banana fruit.

Production of recombinant proteins has led to production of pharmaceutically important peptides such as vaccines in plants (Mason and Arntzen 1995; Rao et al. 2000; Ryu and Nam 2000). Banana is being considered as the most suitable crop for production of edible vaccines for immunization programs. The advantages are that banana fruits are readily available at low cost, can be consumed as raw and their digestibility and palatability by infants is also good. Furthermore, banana being a triploid crop there is no risk of biological containment (Ganapathi et al. 2003). Production of Hepatitis B antigen (HbsAg), in tobacco was first time demonstrated by Mason et al. (1992). They demonstrated that these antigens are equally efficient to yeast derived HBsAg with respect to their ability to raise antibody production in vitro and in vivo (Richter et al. 2000). Studies are in progress for commercial production of edible vaccines against Hepatitis B virus in banana and tomato (Ganapathi et al. 2003). However, standardization of dose in each fruit is necessary.

4.2.2 Cocoa, *Theobroma cacao* L.

Theobroma cacao L., a tropical perennial tree belonging to *Sterculiaceae* family, originated in the Amazon basin (Whitlock et al. 2001). Currently, cocoa is cultivated over 5 million hectares of tropical lowlands worldwide (Soberanis et al. 1999; Duguma et al. 2001; Kraus and Soberanis 2001; Ramirez et al. 2001). *T. cacao* was originally prized by the ancient meso-Americans as an ingredient in ritual drinks (Hurst et al. 2002). The processed form of cocoa seeds, cocoa is now the basis of a multi-billion dollar chocolate industry (Wood and Lass 1987). Trade from dried cocoa beans has an annual estimated value of 2.9 billion US$ per year (Gray et al. 2000).

T. cacao is of high economic significance to many small crop farmers in developing countries. Cocoa beans are roasted and used to produce powder, butter and other commodities of importance to the confectionary and cosmetic industries. Considering the benefits of cocoa as ecologically beneficial agro ecosystems, it is being promoted for environment friendly poverty amelioration (http://www.treecrops.org) (Maximova et al. 2003, 2005).

As cocoa is grown in warm, shady and humid conditions it is susceptible to various pests and fungal diseases which severely affect the yield. Table 4.3 summarizes the common pests and diseases of cocoa. The disease with Cacao swollen shoot virus is described in detail in Chap. 9.

Among these pests and diseases, Cocoa Pod Borer (CPB) pest and fungal diseases like witches broom, black pod and monila pod rot are of major concern. To date, chemical control is the only practiced method to overcome this problem. Unfortunately no complete natural genetic resistance has been observed in cocoa germplasm. Only partial resistance/tolerance has been identified and deployed in some breeding programs over the past century (Eskes and Lanaud 1997).

Table 4.3. Major pests and diseases and its causative organisms in cocoa

Disease	Causative organisms
Pest	Cocoa pod borer
Fungal	
Witches' Broom	*Crinipellis perniciosa*
Frosty Pod/monila pod rot	*Moniliophthora roreri*
Mal de machete or *Ceratocystis* wilt	Ceratocystis fimbriata
Black Pod	*Phytophthora megakyrya*
	Phytophthora palmivora
Viral	
Vascular Streak Dieback	*Onchobasidium theobromae*
Swelling of the root and stems	Swallon shoot virus

Source: World Cocoa Foundation, http://www.chocolateandcocoa.org/Library/Disease/default.asp

The first report on genetic transformation of cocoa appeared in 1994 (Sain et al. 1994). In this study the success was achieved in obtaining transformed callus cells only and there was no regeneration of plants. Since then there was a long gap in gene transformation studies until improved protocol for transformation and regeneration of transgenic plants was reported by Maximova et al. (2003). This group has carried out extensive research on development of gene transfer protocol and could regenerate 94 transgenic plants, by cocultivation of cotyledonary explants from primary somatic embryos (SES) with *A. tumefaciens* strain AGL1. The transgenic plants were successfully transferred to greenhouse and grown to maturity. Detailed growth analysis indicated that there were no differences in various growth parameters between transgenic and non-homogenic somatic embryo derived plants. Seeds produced from two genetic crosses with one of the transgenic lines were analyzed for *GFP* expression – a near perfect 1:1 regeneration was observed, conferring the insertion of a single locus of T-DNA (Maximova et al. 2003).

The major problem in cocoa is susceptibility to various pests and diseases (Table 4.3), which severely reduce its yield by 40% (McGregor 1981; Fulton 1989). Genetic engineering for CPB resistance involves inserting the gene encoding a protein toxic to CPB larvae into the cocoa genome and its expression in pods. Studies to develop transgenic cocoa showing resistance to CPB moth are underway.

To confer resistance to fungal pathogens, Maximova et al. (2003) reported transfer of chitinase gene along with *GFP* to cocoa using *A. tumefaciens*. However, this transformation was not able to confer resistance in transgenic plants towards fungal pathogens. De Mayolo (2003) reported similar results which suggest that expression of chitinase gene does not always give resistance towards fungal disease.

Genetic transformation studies in cocoa are at the initial stages and need special attention. Studies on introducing pest and disease resistance are underway and are likely to be successful in the near future. Besides this, improvement of yield and quality of cocoa beans is also a major concern considering their commercial value in chocolate industry.

4.2.3 Coffee, *Coffea* sp.

Coffee (*Coffea* sp.) is a woody shrub belonging to the family *Rubiacae*. It is commercially cultivated on a large scale for its beans. More than 80 species of coffee are known, and among these the most economically important are *Coffea arabica* L. (Arabica) (2n=44) and *C. canephora* Pierre (Robusta) (2n=22) (Etienne-Barry et al. 2002). Some other species such as *C. liberica, C. dewevrei* and *C. racemosa* are cultivated to satisfy local consumption (Carneiro 1999). Arabica coffee, grown at medium and high altitudes (about 2000 m), is responsible for about 75% of the commercial world coffee and for all the coffee production in Latin America, Ethiopia and Kenya. Robusta coffee is mainly grown in African countries at low altitudes (about 850 m), and 80% of African coffee production is from this species. Robusta has also been cultivated in American and Asian countries (Carneiro 1997).

Coffee is an extremely important cash crop with more than 6.5 million tons of green beans produced every year on about 11 million hectares. In the international markets, it stands second only to petroleum and generates an income of 15 billion US $ per year (Sreenath 2003).

Coffee is known to be susceptible to number of pests and diseases. Some of them are listed in Table 4.4. Among the most damaging pests are leaf miner and coffee berry borer. Fungal diseases that severely affect the yield are coffee leaf rust, leaf spot and root rot.

Genetic transformation studies in coffee are significantly important because of its extreme narrow genetic base, lack of efficient breeding tools/linkage maps, difference in ploidy level, long pre-bearing period and susceptibility to various pests and diseases. Coffee is a self-pollinating plant with selfing being estimated at 90% under plantation condition (Carvalho 1988). Attempts were made to generate better hybrids of coffee by crossing different varieties of *C. arabica*. (Carvalho et al. 1991; Etienne-Barry et al. 2002). Arabica does not have any natural resistance to diseases, and hence these hybrids are not widely accepted. To date 80% of the world's plantations use high yielding hybrid "Hibrido de timor" (HDT) (van der Vossen 2001) which was derived from a natural cross between *C. arabica* (2n=4×=44) and *C. Canephora* (2n=2×=22) (Bettencourt 1973). The phylogenetic analysis using DNA sequencing and molecular marker data revealed extremely reduced genetic diversity in *C. arabica* as compared with *C. canephora* (Anthony et al. 2001).

Table 4.4. Major pests, diseases and its causative organisms in coffee

Diseases	Causative organism
Pests	
White Stem Borer	*Xylotrechus quadripes*
Coffee Berry Borer	*Hypothenemus hampei*
Shot hole Borer	*Xylosandrus compactus*
Leaf miner	*Leucoptera coffeella; Perileucoptera spp.*
Mealybugs	*Planococcus citri & P. lilacinus*
Green Scale	*Coccus viridis*
	Pratylenchus coffeae
Fungal	
Coffee Leaf Rust	*Hemileia vastatrix*
BlackRot	*Koleroga noxia*
Brown root disease/Stump rot	*Fomes noxius*
Red Root Disease	*Poria hypolateritia*
Black Root Disease	*Rosellinia arcuata h*
Santavery Root Disease	*Fusarium oxysporum f. sp. Coffeae*
Die-back	*Colletotrichum gleosporoides*
American leaf spot	*Mycena flavida*
Tracheomycosis wilt	*Fusarium xylorioides*
Collar-rot	*Rosellinia bunodes*
Root rot	*Armillaria mellea*

Source: Central Coffee Research Institute, India

Initially almost a decade was devoted to the development of a suitable protocol for genetic transformation and regeneration of coffee. Spiral and Petiard (1991) reported genetic transformation in coffee for the first time. This report described successful transformation of coffee protoplasts using different strains of *A. tumefaciens* carrying *NPTII* and *GUS* genes under the control of *CaMV35S* promoter to produce transformed callus. The gene transfer studies were carried out using protoplasts, somatic embryos, hypocotyls of in vitro germinated seeds, embryogenic callus, suspension cultures and microcuttings. Although different gene transfer techniques such as electroporation (Barton et al. 1991; van Boxtel 1994), particle bombardment (van Boxtel 1994), and direct DNA uptake (de Pena 1995) have been tested in coffee, *Agrobacterium* mediated gene transfer has been used most widely (Ocampo and Manzanera 1991; Spiral et al. 1993; Grèzes et al. 1993; Gimenez et al. 1994).

A. rhizogenes transformation was first time reported by Spiral et al. (1993). The results of this study were encouraging with 10–40% efficiency. The

transgenic plants when transferred to greenhouse exhibited disrupted phenotypes like crinkled leaves, short internodes, etc. Among tested coffee species *C. canephora* showed more distorted phenotype (Spiral et al. 1999). This protocol was further modified with the use of disarmed *A. tumefaciens* and the transgenic *C. canephora* plants exhibited normal phenotype. Studies on successful incorporation of hygromycin resistance gene *HTP* (Hatanaka et al. 1999) and herbicide chlorsulfuron resistance gene along with *GUS, NPTII* have been reported using *A. tumefaciens* (Spiral et al. 1999).

In view of the fact that no complete natural resistance has been identified in widely cultivated arabica coffee, the strategy of transfer of genes of non-plant origin for providing resistance towards pests and diseases has been successfully adapted. To incorporate resistance towards leaf miner pest, Leroy et al. (2000) transferred *cry1AC* gene from *Bacillus thuringienesis* in both *C. arabica* and *C. canephora* using *A. tumefaciens*. However, the efficiency of transformation was low in *C. arabica* as compared to *C. canephora*. It may be attributed to the low embryo regeneration efficiency in *C. arabica*. Out of 23 plants tested, 18 plants showed expression of *cry1AC* protein in the leaves and molecular analysis confirmed the presence of a copy of T DNA in transformed plants. Bioassays with two different leaf miner species confirmed the expression of *cry1AC* protein and observed resistance at three levels in transgenic plants as highly resistant, slightly susceptible and fully susceptible. Field trials are in progress (Leroy et al. 2001).

Considering the needs of consumer market, manipulation of caffeine content is of great significance in crop like coffee. Caffeine is an important alkaloid present in coffee that is used in beverages (Mizuno et al. 2003). The caffeine biosynthetic pathway has been well studied and consists of three steps of *S*-adenosyl-L-methionine (SAM)-dependent methylation of xanthine derivative (Ashihara and Crozier 1999, 2001). Three distinct *N*-methyltransferases involved are xanthosine methyltransferase (*XMT*), 7-*N*-methylxanthine xmethyltransferase (*MXMT*, theobromine synthase), and 3,7-dimethylanthine methyltransferase (*DXMT*, caffeine synthase) (Ogita et al. 2004). Uefuji et al. (2003) isolated cDNAs designated as *CaXMT1, CaMXMT1, CaMXMT2* and *CaDXMT1* and showed that caffeine can be synthesized in vitro by the combination of these gene products. Ogita et al. (2004) suppressed expression of *CaMXMT1* in *C. arabica* and *C. canephora* by double stranded RNA interference (RNAi). Using *A. tumefaciens,* double stranded RNA sequence of *CaMXMT1* was transferred to single embryogenic cell line derived from a leaf section of mother tree of *C. arabica* and somatic embryos of *C. canephora*. The RNAi transgenic lines of embryogenic tissues derived from *C. arabica* and transgenic plantlets of *C. canephora* demonstrated a clear reduction in theobromine and caffeine content (up to 30–50%) as compared to control plants (Ogita et al. 2004).

Although a number of reports are available, transformation and production of transgenic coffee plants is still viewed as problematic due to time required for the process (Carneiro 1997). It takes approximately 12–20

months from primary inoculation of explants to plantlet transfer to greenhouse (Spiral et al. 1999). Furthermore, the process is delayed because the flowering in coffee is expected four years later (Etienne et al. 2002). The continuous progress in enhancing transformation/regeneration efficiency, successful attempts to introduce disease resistant genes and manipulation in caffeine content are bound to change the scenario of coffee genetic improvement.

4.2.4 Eucalyptus, *Eucalyptus* sp.

Eucalyptus is a large genus of family *Myrtaceae* comprising of evergreen aromatic trees indigenous to Australia, Tasmania, New Guinea and neighboring islands. Eucalyptus must have been known from the early sixteenth century when the Portuguese colonized Timor. There are at least two indigenous species, *E. alba* and *E. urophylla* on the island. *Eucalyptus* came into recorded history in 1788 when the French botanist, L'Héritier de Brutelle, published *Eucalyptus oblique* (Brooker et al. 2002).

E. camaldulensis, E. globulus, E. grandis, E. gunnii, E. urophylla, and *E. tereticornis* (Mysore hybrid) are important species of eucalyptus. *E. camaldulensis* (river red gum) is the world's most commonly planted species in arid and semi-arid climates and possibly the second most important species of eucalyptus in the world in terms of current annual increment of wood (Eldridge et al. 1993).

Eucalyptus is the source of timber and fiber which forms basis of massive paper industries. They contain oils which have several medicinal properties.

Eucalyptus is susceptible to various pests and diseases as shown in the Table 4.5. Defoliating attack by beetles is very damaging in Eucalyptus.

Conventional breeding of eucalyptus is limited by high level of heterozygosity, long breeding cycles and large segmented populations (Teulieres et al. 1994; MacRae and van Staden 1999). The gene transfer studies in *Eucalyptus* are important (i) to confer resistance towards insects, pests and pathogens, (ii) to develop herbicide resistance, (iii) to improve the quality of pulp and (iv) to understand molecular basis of wood formation.

Several reports are available on genetic transformation and regeneration of transgenic eucalyptus plants and their field trials. Although initially different gene transfer techniques have been tested *Agrobacterium* mediated transformation has been preferred. The first report on eucalyptus gene transfer was through electroporation of protoplasts of *E. gunnii* using polyethylene glycol (PEG) (Teulieres et al. 1991) and *E. citridora* (Manders et al. 1992). In these studies only transient expression was observed and no transgenic plant was produced. The biolistic transformation was reported by Rochange et al. (1995) and Serrano et al. (1996). Rochange et al. (1995) obtained transient *GUS* expression in six-day-old *E. globulus* embryos. However, there was no regeneration of plants. Serrano et al. (1996) used

Table 4.5. Major pests, diseases and its causative organisms in eucalyptus

Disease	Causative organisms
Insect/pests	
Defoliation	Scarab beetles (*Anoplognathus* sp.)
Sap feed	Psyllids (*Cardiaspina* sp.)
Defoliation	Leaf beetles (*Chrysophtharta & Paropsis* sp.)
Wood feed	Subterranean termites (*Coptotermes* sp.)
Borers	Wood moths (*Endoxyla* sp.)
Defoliation	Spring beetles (*Liparetrus* spp.)
Fungal	
Little leaf	*Phytoplasma*
Cylindrocladium leaf spot and blight	*C. quinqu`eseptatum; C. scoparium; C. candelabrum*
Eucalyptus rust	*Puccinia psidii*
Mycosphaerella leaf spot	*Mycosphaerella molleniana* and *M. cryptica*
Blight spots	*Cryptosporiopsis eucalypti*
Kirramyces leaf spot	*Kirramyces epicoccoides; Phaeoseptoria eucalypti; K. lilianiae, K. eucalypti, Cercospora eucalypti*
Aulographina leaf spot	*Aulographina eucalypt; Lembosiopsis eucalyptina; Thyrinula eucalypti*
Bacterial	
Bacterial wilt	*B. solanacearum*
Botryosphaeria canker	*Botryosphaeria dothidea; Fusicoccum aesculi; B. rhodina; Lasiodiplodia theobromae; B. theobromae; B. appendiculata*
Cryphonectria canker	*Cryphonectria cubensis, Diaporthe cubensis*
Endothia canker	*Endothia gyrosa*
Coniothyrium canker	*Coniothyrium eucalypticola, C. ahmadii, C. kallangurence, C. ovatum,* and *C. parvum*
Pink disease	*Corticium salmonicolor*
Viral	
–	Mosaic virus

Source: Pacific Forest Health Workshop, Suva, 31 March–3 April 2003, and Country Report – Australia

zygotic embryos of *E. globulus* and were the first to report stable transformation for two months. Mullins et al. (1997) and Ho et al. (1998) reported successful regeneration and transformation in *E. camaldulensis* using leaf explants from *A. tumefaciens*. Mullins et al. (1997) tested the same regeneration protocol for other eucalyptus species like *E. grandis, E. ochrophloia,*

E. marginata and *E. microtheca* but the regeneration frequency observed was low compared to *E. camaldulensis*. Transformed tissue was obtained in five clones of *E. camaldulensis* but only one clone regenerated to whole plant (Mullins et al. 1997). Machado et al. (1997) studied *Agrobacterium* strain specificity in eucalyptus hybrid (*E. grandis* × *E. urophylla*). In this study 5 *A. rhizhogenes* and 12 wild type *A. tumefaciens* strains were evaluated for their virulence and it was found that each strain was showing different degree of virulence. *Agrobacterium* mediated transformation studies in *E. globulus* were reported by Moralejo et al. (1998), Bandopadhyay et al. (1999) and in *E. nitens* by Bandopadhyay et al. (1999). The efficiency of *Agrobacterium* mediated gene transfer has been improved by pre-sonication of germinating seeds (Gonzalez et al. 2002). Llewellyn (1999) for the first time successfully introduced the glyfosate resistance gene in *E. grandis* and generated transgenic plants. These plants were successfully transferred to the field, which is well known as Shell Forestry.

E. camaldulensis is known to be susceptible to insect pest Tasmanian eucalypt leaf beetle, *Chrysophtharta bimaculata* (Elliott et al. 1993) which causes defoliation resulting in significant reduction in wood production and extending plant rotation time. It has been shown by Harcourt et al. (1996) that the first instars of two native chrysomelid species are susceptible to *Cry3A* protein. Harcourt et al. (2000) introduced this gene to *E. camaldulensis* under the strong promoter from pea plastocyanin gene (*petE*) (Pwee and Gray 1993) involved in photosynthesis. Transgenic plants expressed *cry3A* gene in green chloroplast containing cells of leaves conferring resistance to defoliating insects. Two transgenic lines were developed which showed expression of *Cry3A* protein at 0.0025% to 0.01% of total soluble proteins and this has provided resistance to early instars of one chrysomelid species as *C. variicolis*. Another transgenic line exhibited resistance towards *C. bimaculata* and *C. agricola*. Bioassays were performed to detect the mortality rate of various insect species. In this study, *BAR* gene, which is responsible for tolerance to the broad-spectrum herbicide glufosinate ammonium or phosphophinothricin (PPT), was used as selectable marker. The transgenic lines obtained exhibited resistance towards broad-spectrum herbicide Liberty® at 6 l/ha (1.2 kg active ingredient per hectare).

Considering the commercial importance of eucalyptus in paper industry, the main thrust is to reduce lignin content. This can be achieved by down-regulation of different genes involved in lignin biosynthesis. Extensive work has been done on lignin modification in other tree species (for review see Boudet 2000 and Chap. 5). Reports on lignin reduction indicate that different enzymes from lignin biosynthetic pathways have been manipulated. These include cinnamyl alcohol dehydrogenase–CAD (Baucher et al. 1996), cinnamte 4-hydroxylase–C4H (Sewalt et al. 1997) and hydroxycycinnamate CoA ligase–4CL (Kawaoka et al. 2003). In *E. camaldulensis*, C4H gene from *Populus tremuloides* was successfully introduced by Chen et al. (2001) using *A. tumefaciens*. Clonal propagules were raised through rooting of cuttings

and analyzed for presence of transgene. Stable integration of C4H gene was observed in all of the transgenic plants. Large scale multiplication of these transgenic plants by rooting of cuttings have been successful and plantlets have been planted in Liukuei Experimental Forest, Taiwan Forest Research Institute (TERI) and their growth performance is being tested. Valério et al. (2003) reported transformation of *E. camaldulensis* with CAD antisense cDNAs resulting in significant down regulation of its activity. Tournier et al. (2003) and Kawaoka et al. (2003) also suggested similar strategy for down-regulation of gene expression. Kawaoka et al. (2003) identified a cDNA encoding transcription factor, *Ntlim1*, which specifically binds to phenylalanine ammonia lyase (PAL) -box, from *Nicotiana tabacum*. This antisense copy of *Ntlim1*, when transformed into tobacco, indicated 27% reduction in lignin content. Further antisense copy of *Ntlim1* along with kanamycin resistance gene was transferred in *E. camaldulensis* and transgenic plants were grown in greenhouse. There was no abnormal growth in any of the 45 transformed lines obtained. About 20% reduction in lignin content and increase in holocellulose was observed.

Studies on wood formation in eucalyptus are in progress (for review see Leitch and Savidge 2000). Recently the molecular basis of wood formation under controlled laboratory conditions has been attempted by Antanas et al. (2005). In this study, apical stem segments of *E. globulus* were transformed using *A. tumefacians* and stable transformation events were observed at sectors of transformed tissue derived from primary transformation events in individual cells. This system is valuable for the analysis of gene function during the process of wood formation and wood quality determination as well as for constructing developmental fate maps of cambial derivatives (Antanas et al. 2005).

4.2.5 Oil Palm, *Elaeis guineensis* Jacq.

Elaeis guineensis Jacq., a member of family *Palmae*, is the most important species in the genus *Elaeis*. It is the second largest supplier of fats and oils in the world after soybean and sunflower (Scowcroft 1990).

The oil palm is indigenous to West Africa (Hartley 1988). European explorers discovered the palm in the late 1400s, and distributed it throughout the world during the slave trade period. Four oil palm seeds were brought to a Javanese botanical garden in 1848, and the resulting progeny formed the basis of the world's largest production region. The 'Deli' cultivar was discovered near Deli, on the island of Sumatra, and had a much higher oil yield due to its relatively thick mesocarp. The first plantations were established in Sumatra in 1911, and in 1917 in Malaysia. Oil palm plantations were established in tropical America and West Africa about this time. In recent times, palm oil production equalled that of soybean, which had been the number one oil crop for many years. The second species is *Elaeis oleifera* (H.B.K)

Cortes also known as American oil palm which is found in South and Central America (http://www.uga.edu/fruit/oilpalm.htm)

The establishment of new plantations in South East Asia is predicted to double oil palm production in the next 15 years, making it, by then, the most important source of vegetable oils in the world (Murphy 1999).

Conventional breeding in oil palm is limited due to time constraint as it has a comparatively long generation time (seven to ten years), an open pollinating nature and narrow gene pool of present planting material. Genetic engineering can save 80–90% of the time required by conventional breeding for introducing a new gene into oil palm (Parveez 1998).

The genetic transformation in oil palm was first demonstrated more than ten years ago. Reports on genetic transformation of oil palm indicate that *Agrobacterium* mediated transfer is less efficient than biolistic transformation (Abdullah 2005). Initially, the protocol for DNA delivery to embryogenic calli was optimized but only transient *GUS* expression was observed (Parveez et al. 1997, 1998). Studies were also performed to analyze the activity of different promoters and better selective agents (Chowdhury 1997; Parveez et al. 1996). Roberts et al. (1997) transformed immature embryos (age 11–12 weeks after anthesis) using *Agrobacterium* and demonstrated that the efficiency of transformation was improved by sonication, which resulted in 30–45% increase in transient expression. However, the efficiency of stable transformation remained low and inconsistent.

In Table 4.6 an overview about pests and pathogens that commonly affect oil palm is given. Initially efforts were made to introduce genetically insect/pest/disease resistance and herbicide tolerance genes into oil palm whereas, recently, transformation studies have been focused on manipulating the metabolic pathways involved in fatty acid biosynthesis. Another important emerging area is the production of bioplastics in oil palm (Abdullah 2005).

Cowpea trypsin inhibitor (*CpTI*) gene was first used for the transformation of oil palm to increase its tolerance to bagworm larvae (*Metisa plana* Walker). Transgenic plants were found to be tolerant to bagworm larvae as compared to non-transformed plants. The first batches of transgenic plants produced are now more than eight years old. The stability of transgene integration was continuously monitored using molecular techniques. Transgenes were detected in both reproductive and non-reproductive organs of T_0 plants. Using the same strategy, but with different gene(s), work is in progress to address the basal stem rot caused by *Ganoderma boninense* in oil palm (Abdullah 2005).

The main thrust of genetic engineering of oil palm is to manipulate its fatty acid content, thereby improving its quality and utility. Palm oil obtained from mesocarp of oil palm, *E. guineensis* Jacq. Tenera, contains 44% palmitic acid (Rajanaidu et al. 1997). Research indicates that a relatively high percentage of palmitic acid in mesocarp of oil palm is in part due to PATE enzyme

Table 4.6. Major pests, diseases and its causative organisms in oil palm

Disease	Causative organisms
Pests	
Pests (in nursery)	Slugs and snails, Red spider mites (*Oligonchus* sp.), grasshoppers, Cockchafers (*Apogonia* and *Adoretus* sp.), crickets and rats (*Rattus* sp.)
Pests (in field)	Leaf-eating caterpillars, rhinoceros beetle, moths (*Tirathaba*), and porcupines (*Hystrix brachyura*)
Bagworm	*Mahasena corbitti, Metisa plana* and *Cremastopsyche* spp.
Nettle caterpillars	*Setora nitens, Darna trima* and *Thosea asigna*
Adult beetles	*Oryctes rhinoceros*
Fungal	
Leaf spot	*Curvularia eragrostides, Helminthosporium sp*. and *Corticium solani*
Seedling blast	*Pythium* sp.
Basal stem rot	*Ganoderma boninense*
Spear, bud and upper stem rot	*Phellinus noxius*

Source: http://www.sabah.gov.my/tani/english/crop_oil_palm.htm

known to terminate fatty acyl group extension via hydrolyzing an acyl group in palmitic acid. High-stearate, high-oleic, high-linoleic, or low-palmitate oil palms can be obtained by employing DNA recombinant technology (Liu et al. 2002; Thelen and Ohlrogge 2002). Different key genes, kernel specific genes and promoters involved in oil palm fatty acid biosynthesis have been isolated, which can be used to manipulate fatty acid biosynthesis at different levels (Shah and Asemota 2000; Shah and Hanafi 2000; Shah and Cha 2000, 2003; Shah and Rashid 2000; Shah et al. 2000; Cha 2001; Cha and Shah 2001). Two approaches were considered to produce more oleic acid, one of those being to increase the level of a ketoacyl carrier protein (ACP) synthase II (*KAS II*). Efforts were undertaken to isolate the genes involved in oleic acid production. Parveez et al. (2000) proposed the transformation of oil palm with a sense copy of *KAS II* and an antisense copy of palmitoyl-ACP thioesterase under the control of a mesocarp-specific promoter for the production of more oleic acid. The second approach is to reduce thioesterase activity towards palmitoyl ACP. Oil palm contains an active D9-stearoyl-ACP desaturase (D9SAD), which effectively desaturates stearoyl-ACP into oleoyl-ACP. Therefore, by introducing an antisense copy of the D*9SAD* gene, stearic acid could accumulate in the oil palm. The D*9SAD* gene has been isolated from oil palm (Abdullah et al. 1997). Construction of transformation vectors carrying a partial antisense copy of *D9SAD* gene has been successful. Transformation of oil palm callus with these constructs was being carried out (Parveez et al. 2000).

Production of polyhydroxy butyrate (PHB) in plants makes it possible to produce PHB on a large scale. Three enzymes, namely 3-ketothiolase, acetoacetyl-CoA reductase and PHB synthase are known to form PHB in bacteria (Anderson and Dawes 1990). Efforts were made to introduce these genes into oil palm, which may lead to the accumulation of PHB in oil palm tissues. The studies have already been initiated (Moffat 1999). Although different strategies have been suggested for oil palm improvement, the further reports on the progress on these lines could not be obtained.

The transformation studies in oil palm are at the beginning stage. The isolation of target genes and promoters will accelerate this research. However a major difficulty that needs to be resolved urgently is "mantled phenotype formation", i.e. floral phenotypic abnormality observed in oil palm when regenerated through tissue culture methods (Tan et al. 2003). Better regeneration system and enhanced efficiency of stable transformation will help to develop genetically engineered oil palm.

4.2.6 Rubber Tree, *Hevea brasiliensis* Muell. Arg.

Hevea brasiliensis Muell. Arg., a member of the family *Euphorbiaceae,* is one of the major sources of natural rubber. *Hevea* is a heterozygous woody perennial with juvenile period of about six years. This tree species is native to the Amazon basin and was introduced into the Old World at the end of the nineteenth century. All the clones cultivated today are called as Wickham clones, and came from a few seeds collected in 1876 by H. Wickham in a small location near Tapajos River in Brazil. More recently, international surveys were carried out in the Amazon basin in order to broaden the genetic base of domesticated material. Use and management of this germplasm was rendered difficult due to lack of knowledge on the genetic organization of rubber tree (CIRAD 2003).

Although rubber is produced by about 2000 plant species (Backhaus 1985), *H. brasiliensis* (Wild. ex A. Juss) Mull. Arg. (the Brazilian rubber tree) is the only species that produces commercially viable quantities of high quality rubber, i.e. 30 – 50 vol.% of fresh latex (Ko et al. 2003). About 90% of natural rubber is produced in South East Asia (Montoro et al. 2003), of which a major share of rubber production (60–70%) is consumed for the manufacture of pneumatic tires each year.

Hevea is affected by several pests and diseases, particularly *Corynespora* leaf fall. Some of them are listed in Table 4.7.

H. brasiliensis produces a large amount of latex which is the cytoplasm from specialized cells known as laticifers (Dickenson 1969). The latex is present at various locations like intercellular spaces of the roots, stems, or leaves depending on the plant species. In *H. brasiliensis* latex is produced in vessels within the inner bark. The latex is drained from the tree by making a shallow

Table 4.7. Major pests, diseases and its causative organisms in rubber tree

Disease	Causative organisms
Fungal	
White Root Disease	*Rigidoporus lignosus*
Pink disease	*Corticium salmonicolor*
South American leaf blight	*Microcyclus ulei, Fusicladium macrosporum, Aphosphaeria ulei*
Corynespora Leaf Fall (CLFD)	*Corynespora cassiicola*
Colletotrichum Leaf Fall	*Colletotrichum gleosporioides, Glomerella cingulata*
Red root disease	*Ganoderma philippii*
Collar rot 2	*Sclerotium rolfsii (perfect stage Corticium rolfsii Curzi)*
Target leaf spot 3,4	*Sclerotium rolfsii (perfect stage Corticium rolfsii Curzi)*
Brown Root Disease	*Phellinus noxius*
Black Crust	*Phyllachora huberi*
Black Stripe	*Phytophthora palmivora, P. meadii Mc Rae, P. botryosa Phytopthera sp.*
Abnormal Leaf Fall	*Phytophthora palmivora, P. meadii, P. botryosa*
Leaf fall	*Corynespora cassiicola, Cylindrocladium quinqueseptatum, Fusicoccum, Guignardia*

Source: The International Rubber Research Institute http://www.irrdb.com

groove of about 1 mm so as not to cause any damage to the tree and thus allowing a productive life span of up to 15 years. Natural rubber consists of long chains of *cis*-polyisoprene which are synthesized through mevalonate pathway from acetyl *CoA* derived from glycolysis (Keckwick 1989). The polyisoprene chains are grouped together and form aggregates called as rubber particles surrounded by a lipoproteic membrane (Chrestin et al. 1997). In *Hevea* species, latex yield is a clonal character (Jacob et al. 1989). The rubber producing latex cells have been extensively studied at physiological and gene expression levels and a number of determining factors controlling natural rubber production have been characterized (Montoro et al. 2003).

Ethylene plays a major role in enhancing the latex production. Studies have been carried out to identify genes involved in rubber production and their relation with ethylene. Kush et al. (1990) reported the differential gene expression in response to ethylene treatment. Further studies indicated that there is increase in the expression level of some genes after ethylene stimulation (Broekaert et al. 1990; Pujade-Renaud et al. 1994). Chrestin et al. (1997)

studied in detail the molecular basis of latex coagulation and demonstrated that three proteins, namely hevein, hevein receptor and chitinase, were responsible for coagulation of latex. Hevein induce the latex coagulation by bringing two rubber particles, ethylene induces over-expression of three genes coding for hevein, its receptor and chitinase. Chitinase partially deglycosylate the hevein receptor so as to delay rubber coagulation. These studies are extremely important and provide an insight into the mechanism of latex production at molecular and biochemical level and will be useful to improve the potential yield and prolong the latex flow. Shin et al. (1999) isolated a HvDnaJ, a DnaJ homologue (eukaryotic protein assumed to play major role in protein folding and translocation into organelles) from cDNA library of *H. brasiliensis* and studied its expression pattern. Recently the number of expressed sequence tags (ESTs) expressed in the *Hevea* latex have been isolated (Han et al. 2000). Further screening of the ESTs in the latex and their putative role in latex production was investigated (Ko et al. 2003).

The rubber tree is primarily transformed to enhance latex production. The genetic transformation system for *H. brasiliensis* was first reported by Arokiaraj et al. (1991) using *A. tumefacians* (strain 541). The tumor development was observed on stems of in vitro and in vivo propagated seedlings and exhibited phytohormone-independent growth. Particle bombardment has also been successfully attempted by Arokiaraj et al. (1994). In this study callus derived from anthers was used for transformation. Montoro et al. (2000) demonstrated that $CaCl_2$ plays an important role in increasing transformation efficiency of *Agrobacterium* mediated transformation in *H. brasilliensis*. However, detailed studies are necessary to identify the rate limiting factors in transformation.

Another distinct benefit from rubber transformation is the enhancement in oxidative stress tolerance. Frequent rubber tapping for its harvest leads to development of high oxidative stress which causes a physiological disorder known as Tapping Panel Dryness (TPD) or bast syndrome (Jayashree et al. 2003). The excessive level of NAD(P)H oxidase is noticed which ultimately leads to the formation of reactive oxygen species like superoxide (toxic forms of oxygen). The superoxide inhibits latex production by damaging the integrity of lutoid membranes (Chrestin 1989). Superoxide dismutase (SOD) enzyme present in the latex provides defense against oxidative stress in plants. Successful development of gene transfer and regeneration system for *Hevea* enabled to introduce superoxide dismutase gene, *HbSOD*, to *H. brasiliensis* via *Agrobacterium* mediated transformation and transgenic plants were regenerated which are showing constitutive expression of *HbSOD* gene. Currently these plants are growing in polybags. The oxidative stress tolerance will be evaluated when these plants will be transferred to field (Jayashree et al. 2003).

By taking advantage of high latex production capacity of rubber tree, the continuous synthesis of foreign proteins can be achieved in the rubber latex provided that these are transformed with desired genes. Arokiaraj et al.

(1998) demonstrated *GUS* expression in the latex. This report opened the avenue for the continuous production of foreign proteins in the latex. At present the transformation system has been developed for the *Hevea* GL 1 clone, which is not much agronomically valuable (Montoro et al. 2003). Using this approach successful synthesis of a mouse antibody fragment in the latex of transgenic rubber tree has been demonstrated by Yeang et al. (2002). In this study, a gene construct for an antibody single chain variable fragment (scFV) against the coat protein of the bacterium *Streptococcus gordonii* under the promoter *CaMV35S* and nos terminator was used. The proteins thus synthesized are functional, i.e. without loss of their activity. The recombinant GUS protein was expressed when supplied with substrate, while the antibody fragment was found immuno-reactive to its matching antigenic protein. Another report demonstrated the production of human serum albumin in latex of transgenic *Hevea* (Arokiaraj et al. 2002).

In the context of success obtained in rubber transformation, the full outcome of all the above studies indicates steady progress in rubber tree transformation. However, there is a need to study disease resistance, particularly *Corynespora* leaf disease. Refinements of gene transfer protocol to enhance efficiency of transformation will also accelerate rubber tree research. Detailed studies on latex cell specific promoters are needed in order to limit transgene expression to the rubber-producing tissues. The work on cloning of promoters of interest like ethylene inducible promoters for ethylene induced overexpression of transgenes to stimulate rubber production will definitely upgrade rubber productivity in future.

4.3 Conclusions

Modern biotechnological methods have led to successful genetic transformation and production of transgenic trees. The most studied tree species is poplar, because it offers advantage of small genome size, followed by *Pinus* and *Picea*. The commonly used methods of transformation are *Agrobacterium* mediated and biolistic transformation. *Agrobacterium* mediated gene transfer is preferred for tree species because the frequency of obtaining chimeric plants and rate of insertion of multiple number of copies is low; this avoids gene silencing (Mullins et al. 1997). Somatic embryos, embryogenic cell suspension and protoplasts are the candidates for successful transformation and regeneration. Hypocotyls, zygotic embryos, leaf disc and shoot tips have also been used successfully.

The most common traits for which genetic modification can be realized in the near future includes herbicide tolerance, resistance to insects, pests and diseases (nematode, fungal, bacterial and viral) and lignin content. Although transfer of desired gene is a routine, selection of transformed cells and regeneration of transgenic plants is critical. Precise information on the factors

influencing the transformation, regeneration of transgenic plants and stable integration of gene of interest is the key to successful commercial exploitation of these studies as is the reality in the herbaceous crops. Considering the long regeneration cycle of tree species, stable integration and expression of gene is very important, and therefore long-term field trials are essential. Also the analysis of potential benefits and risks of using transgenic trees should be done very critically.

Studies on genetic transformation indicate that in the initial years focus was on development of efficient systems for transformation and regeneration. The most commonly used reporter gene was *GUS* along with the marker genes as *NPTII, HPT, CAT, KAN*, etc. driven by *CaMV35S, ubi1, uidA* promoters. In recent years *GFP* has come up as reporter gene. The work is in progress on tissue specific, and inducible promoters.

Among the tree or tree-like species reviewed in this chapter, banana, coffee and cocoa lack natural resistance towards diseases. Partial resistance has been identified in some species but is insufficient to combat the severe attack of pest and diseases. Hence incorporation of disease-resistant genes from different sources is an alternative for such species. In banana, introduction and expression of antimicrobial peptide (MSI-99) is a milestone in conferring fungal disease resistance (Chakrabarti et al. 2003). Partial resistance has also been developed towards nematodes by integration of oryzostatin gene from rice and further studies to impart complete resistance are under way. A similar strategy is being adopted for cocoa using proteinase inhibitor and chitinase genes (Maximova et al. 2003). Success has been achieved to incorporate *cry1A* gene from *B. thuringiensis* to confer resistance against the leaf miner in coffee (Leroy et al. 2000). Insect and herbicide resistant plants have been developed in eucalyptus (Harcourt et al. 2000).

Understanding the metabolic pathways leading to the production of a commercially important end product is useful in selection of suitable genes for transformation. Metabolic pathways of lignin, caffeine, latex and fatty acid biosynthesis have been studied extensively leading to identification of regulatory steps/enzymes/genes. The information generated is extremely important to control the expression of the key genes. Down-regulation of the enzymes by incorporating antisense copies of genes involved in lignin biosynthesis has been done in eucalyptus and transgenic plants have been produced and field planted (Chen et al. 2001; Valério et al. 2003; Kawaoka et al. 2003). A report on introduction of antisense copy of gene involved in caffeine biosynthesis (Ogita et al. 2004) is a step towards reduction in caffeine content in coffee. Enhanced latex formation in rubber and manipulation of seed oil content in oil palm are the foremost objectives for genetic transformation studies in these trees. Studies on these lines are at primary stage.

A beginning has been made in improving the shelf life of banana fruit (Liu et al. 1999). The successful production of pharmaceutically important peptides in banana appears promising. However, work is needed to develop cold- and drought-tolerant banana to cope with various agro-climatic conditions. The

molecular basis of wood formation in eucalyptus is being studied which will have a definite impact on improving the quality of wood (Antanas et al. 2005). *Hevea* is emerging as an ideal candidate for molecular farming. Synthesis of active mouse antibody and human serum albumin in the latex has already been demonstrated (Arokiaraj et al. 2002). This has opened the way to produce different immunologically and pharmaceutically important proteins in *Hevea*. Furthermore, superoxide dismutase gene has been introduced in *Hevea* and expected to increase tolerance to oxidative stress (Jayashree et al. 2003). The problem of *Corynespora* leaf disease in *Hevea* needs to be addressed.

The major issue about outplanting transgenic plants is biosafety. The escape of transgene in the natural environment is the main limitation for commercialization of transgenic plants. Transgenic trees can easily disperse their pollen and seeds and crossbreed with native trees. Sterility and delayed flowering is considered important for forest trees. A recent report on prevention of flower development in silver birch (Lannenpaa et al. 2005) opened a way to reduce the risk of biological containment via escape of transgenes by inducing sterility, using *BpFRUITFULL-LIKE1* (*BpFULL1*, formerly known as *BpMADS5*) for tissue specific ablation of inflorescences.

In the context of conservative consumer market programs on improving crops like coffee, cocoa, banana, etc., the acceptance at consumer level and hazardous effects of transgene should be considered. Considering the time frame required and cost involved for the production of transgenic trees, to be of economic value, genetically modified trees must offer unique features that are capable of offsetting the high cost.

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Part B Wood and other Traits

5 Environmental Aspects of Lignin Modified Trees

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5.1 Introduction

The increasing global need for food and fiber results in new demands for the efficiency of wood production and wood products (Fenning and Gershenzon 2002) which has to be attained on the basis of sustainable development. The majority of world wood products still comes from natural and semi-natural forests, but there is a clear trend towards more efficient plantation forestry (Walter 2004). Development of vegetative propagation methods, including cutting technology, organogenesis and, in particular, somatic embryogenesis will yield additional profit for plantation forestry by the exploitation of non-additive genetic variation, by providing more homogenous material and by compensating potential shortage of improved seed stock. However, economically relevant clonal plantation forestry is currently a reality for only a few species, out of which *Pseudotsuga menziesii* Mirbel Franco, *Pinus taeda* L., *Pinus radiata* D.Don, *Populus* spp., *Eucalyptus* spp. and *Picea* spp. are prominent (Sutton 1999a,b; Peña and Séguin 2001; Cyr and Klimaszewska 2002).

Conventional breeding of forest trees is in many cases hindered by long generation times and self-incompatibility mechanisms. Genetic transformation of forest trees has been considered the mechanism to achieve genetic gain when combined with conventional breeding and plantation forestry. There are several traits that show potential for a molecular breeding approach, e.g. reduction of generation time, production of sterile trees, pest or disease resistance, wood formation (including lignin and cellulose engineering), resistance to biodegradable herbicides, durability, phytoremediation of polluted sites and the production of novel chemicals and pharmaceuticals (reviewed by Peña and Séguin 2001; Fenning and Gershenzon 2002; Diouf 2003). Highly complex traits such as wood formation and the shortening of the juvenile phase have been considered the most important ones for achieving gain and for further domestication (Fenning and Gershenzon 2002).

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Tree Transgenesis: Recent Developments

Future challenges will most certainly include an improved understanding of wood formation as well as the characterising the controlling genes with particular respect to their expression and regulation (Hertzberg et al. 2001; Lorenz and Dean 2002; Raes et al. 2003). Lignin biosynthesis is now quite well understood and modifications with regards to lignin content and quality have progressed well (reviewed by Boerjan et al. 2003). This provides an option for some practical applications in a commercial plantation forestry environment.

Together with the technological aspects of lignin modified trees, the potential environmental impacts of lignin modification have to be considered carefully. The environmental concerns on lignin modified trees have originated from views on the specific characteristics of forest trees. Trees are long-living organisms, many of which are wind pollinated. As primary producers they make contributions to community structure and ecosystem processes, such as nutrient and carbon cycles. The recognition of potential risks or benefits associated with the operational use and deployment requires comprehensive analyses in both contained use and field trials. Elegant reviews of perspectives on risk in transgenic forest plantations have recently been published by Burdon and Walter (2001), van Frankenhuyzen and Beardmore (2004) and Walter (2004). So far, there is relatively little information on environmental effects of lignin modified trees (Pilate et al. 2002). In this review we will discuss the information available on the environmental aspects of lignin modified trees with a specific focus on lignin modification in the pulp and paper industry as well as on the ecological interactions with insect herbivores and symbiotic ectomycorrhizal fungi.

5.2 Lignin and Current Knowledge of Lignin Biosynthesis

Lignin is a phenolic biopolymer with several important functions in plants. It is an essential cell wall component of woody plants, representing approximately 15–35% of the dry weight of the trees. It provides stiffness and strength to the stem and enables water and solute transport in the vascular xylem. It has also been considered to have a protective role against pathogens and herbivores (Denton 1998). Lignin is synthesized from aromatic heteropolymers that mainly originate from the oxidative polymerization of monolignols 4-hydroxycinnamyl alcohol, coniferyl alcohol and sinapyl alcohol. These components produce hydroxylphenyl (H), guaiacyl (G) and syringyl (S) lignin units, respectively. In conifers, lignin is mainly composed of G units, while in deciduous tree species both G and S units are involved. Lignin biosynthesis and lignin properties have been the targets of intensive research for more than a century (Sarkanen 1971; Higuchi 1985; Sederoff and Chang 1991; Chiang et al. 1994), mostly because extraction of lignin from wood fibre is an important cost factor for the pulp and paper industry. In general, the efficiency of wood pulping is directly proportional to

the amount of S units in lignin. The G units of gymnosperms have a 5-aromatic position leading to very strong carbon-carbon bonds, which make them fairly resistant to depolymerization required during pulping (Boudet and Grima-Pettenati 1996).

Lignin precursor biosynthesis is certainly the best known metabolic pathway of the phenolic metabolome of plants. During the last decade, a lot of new information has been gathered on this highly complex biosynthetic process (Osakabe et al. 1999; Li et al. 2000, 2001). It has been demonstrated that many enzymatic reactions occur at the level of hydroxycinnamic esters, aldehydes and alcohols (e.g. Osakabe et al. 1999; Hoffman et al. 2004). The current simplified model of lignin biosynthesis in angiosperms is presented, for instance, by Li et al. (2001), Boudet et al. (2003) and Hoffman et al. (2004). Initially, phenylalanine ammonia-lyase (PAL) catalyzes the deamination of phenylalanine to cinnamate, followed by cinnamate-4-hydroxylase (C4H) which produces 4-coumarate. Subsequently, in trees the role of 4-coumarate:coenzyme A ligase (4CL) that catalyzes the reaction from acids to esters (i.e. from 4-coumarate to *p*-coumaroyl CoA) has recently been emphasized (Hu et al. 1998, 1999; Harding et al. 2002). *p*-Coumarate 3-hydroxylase (C3H) with the novel HCT, i.e. an acyltransferase controlling shikimate and quinate ester intermediates accomplish after a series of reactions the synthesis of caffeoyl CoA from *p*-coumaroyl CoA.

In the new concept of lignin precursor biosynthesis in trees, the role of caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) (Meyermans et al. 2000; Zhong et al. 2000) that catalyzes the reaction from caffeoyl CoA to feruloyl CoA, has also been demonstrated. This suggests that the route from caffeate to sinapate is not used for lignin biosynthesis (Osakabe et al. 1999; Li et al. 2001). Subsequently, cinnamoyl-CoA reductase (CCR) catalyzes the reaction from esters to aldehydes (i.e. from feruloyl CoA to coniferaldehyde), followed by cinnamyl-alcohol dehydrogenase (CAD) catalyzing the reaction from aldehydes to alcohols (i.e. from coniferaldehyde to coniferyl alcohol). The conversion of guaiacyl monolignol intermediates into syringyl types may take place at coniferaldehyde (Osakabe et al. 1999; Li et al. 2000). Coniferaldehyde 5-hydroxylase, which is also known as ferulate 5-hydroxylase (Cald5H and F5H, respectively), and 5-hydroxyconiferaldehyde *O*-methyltransferase also known as caffeate/5-hydroxyferulate *O*-methyltransferase (AldOMT and COMT, respectively), catalyze the reactions from coniferylaldehyde to produce sinapylaldehyde. The last step in the syringyl monolignol pathway is catalysed by sinapyl-alcohol dehydrogenase SAD (Li et al. 2001) leading to sinapyl alcohol. However, the syringyl lignin biosynthesis in angiosperms has been found to operate via multiple pathways, depending on the phylogenetic group of the tree species (Yamauchi et al. 2003). The last step in lignin biosynthesis, the oxidation of monolignols, is presumably catalyzed by peroxidases or laccases. Peroxidases can generate phenoxy radicals, and these radicals are coupled into lignin polymers. What is more, the complex issue of linkage specificity during monolignol

polymerization in the cell wall has evoked active discussions between the dirigent protein (Davin and Lewis 2000) and random coupling models (Sederoff et al. 1999; Hatfield and Vermerris 2001; Boerjan et al. 2003).

5.3 Lignin Modification in Genetically Engineered Trees

At present, genetically modified plants or mutants with modified expression of all genes involved in monolignol biosynthesis pathway have been studied in detail (reviewed by Boerjan et al. 2003; Hoffmann et al. 2004). Genetic engineering of lignin has been considered an important tool for providing perspectives on the understanding of lignin biosynthesis, structure and properties as well as for providing economical benefits for the pulp and paper industry. The goal of genetic engineering of lignin has been to modify either lignin content or composition, for example the S/G ratio. In general, it has been shown that plants can tolerate large variations in lignin content and composition (Boerjan et al. 2003). In addition to *p*-coumaryl, coniferyl and sinapyl alcohols, other uncommon monomers can also be incorporated into the lignin polymer, and this copolymerization may result in novel lignin structures (reviewed by Boerjan et al. 2003). This was also true in our own study in which abnormal 5-OH G units were discovered in lignin of transgenic lines that overexpressed *COMT* under the control of a 35S promoter in *Betula pendula* Roth (Aronen et al. 2003). Based on the results with the model plants tobacco (*Nicotiana tabacum*) and *Arabidopsis* as well as *Populus* spp., the main reductions in lignin content have been achieved by downregulation of *PAL*, *C4H*, *4CL*, *C3H*, *CCoAOMT*, *COMT* and *CCR* (reviewed by Boerjan et al. 2003). Recently, the downregulation of cationic peroxidase *FBP1* in tobacco led to a 40–50% reduction in lignin content (Blee et al. 2003) and in *Populus sieboldii* × *P. grandidentata* downregulation of anionic lignin-specific peroxidase *PrxA3a* resulted in up to 20% reduction in lignin content plus changes in lignin composition (Li et al. 2003b). The changes that were found in lignin composition have been more species-, gene- or enzyme-isoform-dependent. For instance, the downregulation of *4CL* in *Arabidopsis* led to a reduction of G units (Lee et al. 1997), whereas in tobacco the corresponding regulation led to a reduction of S units (Kajita et al. 1997). In *P. tremuloides* Michx. the S/G ratio was at the same level as in the control plants (Hu et al. 1999). Quite striking results were reported for an *Arabidopsis F5H* mutant, which had only traces of S units (Marita et al. 1999) and as a result of upregulation of *F5H* in *P. tremula* × *P. alba*, lignin was almost exclusively composed of S units (Franke et al. 2000).

As discussed by Boerjan et al. (2003), reductions in lignin content by downregulation of *C3H*, *CCoAOMT* or *CCR* have been associated with altered growth and phenotypes that may significantly vary according to developmental and environmental conditions. On the other hand,

downregulation of the lignin-specific *4CL* expression in *P. tremuloides* clearly demonstrated that lignin content can be reduced without compromising the structural integrity and growth of trees (Hu et al. 1999; Li et al. 2003a). These are also good examples of studies in which the modification of one cell wall component (one trait) has resulted in alterations of another one, i.e. 45% reduction in lignin content was compromised by 15% increase in cellulose content (Hu et al. 1999; Li et al. 2003a). As reviewed by Boudet et al. (2003), this may suggest potential cross talk and interconnected pathways between cell wall components and emphasize highly regulated carbon channelling between cellulose and lignin biosynthetic pathways.

Future challenges to understand wood formation better and to benefit the forestry cluster will flow from the adoption of information generated through approaches in systems biology and functional genomics (Bhalerao et al. 2003). An improved knowledge of transcriptional and posttranscriptional mechanisms (Bhalerao et al. 2003) as well as a better understanding of how genotypes and phenotypes are linked (Häggman and Julkunen-Tiitto 2006) may also help to plan transgenic approaches to fine-tune wood-formation or wall structures. As pointed out by Boudet et al. (2003), these approaches may benefit from the already existing multiple-gene transformation protocols (Li et al. 2003a) that enable simultaneous modifications of wall quality traits. Information flow from DNA to RNA and further to proteins and metabolites is not co-linear. This, together with the interconnected metabolic pathways found between cell wall components (Hu et al. 1999; Li et al. 2003a) indicate the need to study not only lignin but also other cell wall components as well as environmental aspects of lignin modified trees.

5.4 Environmental Aspects of Processing Lignin Modified Trees in the Pulp and Paper Industry

The world's forest area is estimated to be between 3.2 and 3.9 billion hectares, which equals around 30% of the Earth's land area. Although it is difficult to find a reliable value for worldwide timber consumption (Fenning and Gershenzon 2002), it is undisputed that the pulp and paper industry is one of the major consumers of timber, and thus has a significant influence on global environment and economy. In the production of paper, lignin needs to be removed with minimal damage to cellulose. Residual lignin in the wood fibers causes rapid discoloration and a low brightness level of the pulp (Chiang et al. 1988). Kraft pulping is the most frequently applied chemical pulping method for the production of high-quality paper (e.g. printing and writing papers). It is based on the use of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) to obtain high sulfidity, which enhances the rate of delignification by cleavage of the *β-O*-4 linkages (predominant interunit linkage in native lignin) (Higuchi 1985) and the methoxy groups. High sulfidity leaves the

carbohydrates less degraded because of the lowered cooking time (reviewed by Baucher et al. 2003). The residual lignin in chemical pulp is removed by subsequent bleaching, i.e. with chlorine or chlorine-free chemicals. The removal of lignin and some of the hemicelluloses results in a low yield of pulp (45–55% of the initial biomass) (Baucher et al. 2003). Furthermore, it has a great economical and environmental impact as it requires chemicals and energy and leads to a high load of undesirable waste products. Therefore, genetic engineering of the lignin biosynthesis pathway may help to answer to the growing demand for raw-wood with improved characteristics for papermaking.

Genetically modified trees that have either a reduced lignin content or a modified lignin composition, which is more suitable for chemical pulping, could be interesting in this respect (Chen et al. 2001). Most of the current experience is based on the Kraft pulping simulations of the lignin-modified *Populus* trees grown in the greenhouse or in the field. Based on the experience gained so far, the genetic suppression of *CAD* may result in altered lignin structure with improved pulping properties (Baucher et al. 1996; Lapierre et al. 1999; Pilate et al. 2002). The pulping benefits were observed as improved extractability of lignin (Baucher et al. 1996; Lapierre et al. 1999; Pilate et al. 2002) and increased yield of high-quality pulp (Pilate et al. 2002). The changes were related to the increased frequency of free phenolic groups in lignin, the characteristic that facilitates lignin solubilization and fragmentation during Kraft pulping, as well as to the slight decrease in overall lignin content in the most severely suppressed *CAD* line (Lapierre et al. 1999; Pilate et al. 2002). Evidence of the beneficial Kraft pulping applied with *CAD* suppression was also obtained with transgenic tobacco (O'Connell et al. 2002). In contrast to these results, the CAD-deficient *Pinus taeda* wood was characterized by an increased incorporation of aldehyde subunits making it well suited for milder soda pulping but not for Kraft pulping conditions (MacKay et al. 1999).

Strong down-regulation of COMT activity may lead to substantial decrease in lignin content, which has a positive effect on the pulp yield (Jouanin et al. 2000). On the other hand, concomitant structural alterations in lignin achieved by *COMT* suppression may be less beneficial for wood-pulping properties. The structural changes such as the decreased proportion of S monomers, β-*O*-4 linkages and/or free phenolic units as well as the enrichment of G units and resistant carbon-carbon linkages provide a higher cross-linking degree of lignin and may deteriorate the Kraft cooking efficiency (Lapierre et al. 1999; Jouanin et al. 2000; Pilate et al. 2002). As already mentioned, reduction in the S/G ratio plays an important role in the pulping process and it has been estimated that one unit increase in the S/G ratio of angiosperm lignin can roughly double the rate of delignification (Chang and Sarkanen 1973). Thus, the modification of the genes affecting S monomer synthesis (Li et al. 2003a) may lead to improved delignification characteristics of wood. Consistent with this, the enriched S monomer content in *Populus tremula* × *P. alba* trees over-expressing *F5H*, resulted in increased Kraft

pulping efficiency of two-year-old wood. This result confirmed the potential of F5H transformation in production of wood-pulp with a lowered need for pulping chemicals (Huntley et al. 2003).

In the pulp and paper industry, using trees with less lignin or easily degradable lignin is an environmentally sound option resulting in a lowered requirement for chemicals and energy. Due to the size of the industry and industrial waste streams, even minor modifications in lignin would be economically and environmentally important. Plantation forests with lignin-modified trees could also remove some pressure from natural forests as wood sources. For the future applications of lignin modified trees, long-term field trials are necessary in order to investigate their ecological interactions at the ecosystem level and to discover the potential pleiotropic or epistatic effects of the transgenes. Furthermore, transgenic plantations are a societal and political issue, in which any decisions should be made on the basis of scientific evidence on the potential risks and benefits. In addition, these decisions should be firmly based on a risk assessment that takes all aspects into account, including the risks of not using a new technology. In essence this means that any risk perceived from, or resulting from genetically engineered forest plantations, should be compared with the risks associated with existing and accepted practise. Due to the long rotation time of the forest trees, it is important that long-term visions, indicating how the increasing need of wood will be combined with sound economics and sustainable development, are developed.

5.5 Ecological Interactions of Lignin Modified Trees

5.5.1 Insect Herbivores

As primary producers, trees support a myriad of insect herbivores that depend on their host as a source of food and habitat. Herbivorous insects demand water, amino acids, sugars and minerals from the plant cytoplasm for their growth and reproduction (Scriber and Slansky 1981; Brodbeck and Strong 1987; Mattson and Scriber 1987) and they can in turn influence growth, reproduction and morphology of their host trees (Crawley 1983). Trees, however, defend themselves against herbivores by means of mechanical and chemical defences (Harborne 1988; Lucas et al. 2000). Lignin as an essential and major component of plant cell walls plays a role in the nutritional ecology of both vertebrate and invertebrate herbivore.

The contribution of lignin to resistance of trees against insect herbivores is difficult to assess in context of the overall quality of the tree tissue that is influenced by both nutritive and defensive components. Plant secondary compounds such as phenolics, terpenes, and lignin can negatively impact insect performance by limiting both host choice and host use due to toxic or

antinutritional effects (Scriber and Slansky 1981; Stamopoulos 1988; Slansky and Wheeler 1992). Lignin may form a physical barrier against insects. For instance, lignified stone cells lower the suitability of conifer bark for bark beetles (Wainhouse et al. 1990, 1998). Together with other cell wall components, lignin contributes to leaf 'toughness', which can have a negative effect on defoliating insects (Feeny 1970; Haukioja 2003), but which is a trait difficult to measure (Lucas et al. 2000; Sanson et al. 2001; Iddles et al. 2003). Both cellulose and lignin dilute the nutritional value of the diet, and insect herbivores have evolved with different adaptive strategies to cope with low quality food. For example, insects are capable of compensatory feeding (Slansky and Wheeler 1992; Slansky 1993; Kause et al. 1999); some insects utilize early season foliage that is rich in nutrients and water but low in quantitative defense compounds and lignin (Feeny 1970), whereas some others, such as leaf miners, have specialized in certain plant tissues (e.g. Kimmerer and Potter 1987). Therefore, possible responses to lignin modification may be specific to insect guilds or species. In general, insects are not able to degrade lignin, with the potential exceptions in wood-feeding subfamily of higher termites (Isoptera: Termitidae: Nasutitermitinae) (Cookson 1988; Prins and Kreulen 1991; Breznak and Brune 1994). As pointed out by Hatfield and Vermerris (2001), the plasticity in lignin composition and the ability to form lignin by random coupling may be advantageous in the defense against pathogens. The lack of regularity might have been problematic in the evolution of hydrolytic enzymes in fungi and insects (Denton 1998) and, consequently, favourable for plant protection.

The knowledge of the effects of lignin modification on insect herbivores is scarce. On the other hand, there are numerous studies on the effects of lignin on the digestibility of forage for livestock (Barrière et al. 2003). Enhancing the digestibility of forage and finding alternative and improved forage have been the goals in genetic engineering of forage species (Barrière et al. 2003; Gressel and Zilberstein 2003; Krause et al. 2003). It has been proposed that the digestibility of plant material can be affected by both the quantity and quality (e.g. S/G ratio) of lignin. The condensed and highly cross-linked G lignin, in particular, is predicted to lead to lower degradability and higher protection of plant cell walls than S unit rich lignin (Jung and Deetz 1993; Baucher et al. 1998; Barrière et al. 2003). So far, the results have been contradictory. For instance, an *Arabidopsis* mutant lacking S lignin did not show a change in cell wall degradability (Jung et al. 1999), whereas in other studies reduction in S lignin improved digestibility in tobacco, *Stylosanthes humilis* and *Arabidopsis* (Vailhé et al. 1996; Rae et al. 2001; Goujon et al. 2003).

One could hypothesize that a decreased amount of lignin and/or G units might increase the suitability of trees to insect herbivores. To our knowledge, there are only two reports on interactions between lignin modified trees and insect herbivores. In our own in vitro interaction experiment we fed leaves of *B. pendula* 35S-PtCOMT lines that had reduced S/G ratio to herbivores that commonly feed on birch. Caterpillars of *Aethalura punctulata*, *Cleora*

cinctaria and *Trichopteryx carpinata* (Lepidoptera: Geometridae) and beetles *Agelastica alni* (Coleoptera: Chrysomelidae) and *Phyllobius* spp. (Coleoptera: Curculionidae) did not demonstrate any significant changes in their preference (food choice) or performance (larval growth rate) that could exclusively be related to lignin modification (Tiimonen et al. 2005, Fig. 5.1). It is possible that the quality of lignin is unimportant for herbivorous insects. On the other hand, the result might reflect the complex characteristics related to both nutritive and defensive factors of plant and insect herbivore interactions (e.g. Ossipov et al. 2001; Haukioja 2003). Another report on the interactions between insect herbivores and lignin modified trees is the field experiment of *Populus tremula* × *P. alba*, in which the structural or slight quantitative changes in lignin of the CAD- or COMT-deficient lines did not affect either species diversity of herbivorous insects or the amount of damage to the leaves (Pilate et al. 2002).

Both feeding preferences and the suitability of a diet for a herbivorous insect can be investigated in the laboratory by means of choice tests and measures of growth rate and food consumption of developing larvae combined with measures of other plant traits. Whether results from these fine-resolution experiments scale up to a level of a plantation is another question. Furthermore, experiments with different insect guilds that utilize different parts of the host trees (e.g. stem wood, bark, phloem, leaves, buds, flowers, seeds, cones, xylem fluid, root tissue) demand test material from trees of different ages and sizes. The environmental effects on resistance traits are strong, and trees grown in contained greenhouse conditions are in many respects different from trees deliberately released in the field. In summary, research on ecological interactions calls for field experiments, combined with contained greenhouse and laboratory experiments as well as a modelling approach.

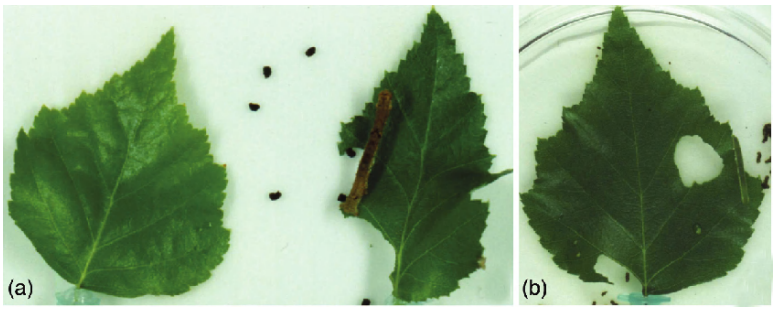

Fig. 5.1(a,b). In vitro herbivory experiments with *Betula pendula* leaves and lepidopteran larvae: (a) two-choice food selection experiment with *Cleora cinctaria* larva. Control leaf is on the *left* and the lignin-modified leaf on the *right*; (b) relative growth rate (RGR) experiment of *Trichopteryx carpinata*

5.5.2 Mycorrhizas

Economically important tree species live in symbiosis predominantly with ectomycorrhizal (ECM) fungi or, as *Populus* species, with both ECM and arbuscular mycorrhizal (AM) fungi. In this unique association the host plant supplies simple sugars to the fungal partner, whereas the fungus improves plant nutrition by increasing the surface that absorbs nutrients and also by enabling the use of organic forms of nutrients (Smith and Read 1997). Ability of certain ECM fungi to use organic forms of N and P is extremely important for the tree species that thrive in acidic and cold boreal forest soils with low decomposition rate (Lindahl et al. 2002). In addition to increased nutrient acquisition, ECM fungi may improve the resistance of the host plant against fungal pathogens that threaten plant roots (Smith and Read 1997).

Ectomycorrhizal fungi, which may colonise even more than 95% of the feeder roots (Taylor et al. 2000), can cause large changes in root morphology. Lateral root formation is induced due to the fungi (Karabaghli-Degron et al. 1998; Tranvan et al. 2000; Niemi et al. 2002, 2005), whereas root hair proliferation is inhibited (Ditengou et al. 2000, 2003). In mature ECMs, feeder roots of the host plant are entirely covered by a hyphal mantle, which isolates the root nutritionally from the soil. Furthermore, a highly branched structure called Hartig net is formed as the fungus grows between epidermal and cortical cells (Smith and Read 1997). The growth of the ECM fungus over and inside the root is controlled both physically and chemically. Weiss et al. (1999) found that in the ECM roots of *Pseudotsuga menziesii* and *Abies alba* Mill. specific phenolic compounds accumulated especially in the inner, non-colonized part of the cortex, probably to restrict fungal growth to endodermis. In contrast, in *Picea abies* Karst (Münzenberger et al. 1990), *Larix decidua* Mill. (Münzenberger et al. 1995), and *Fagus sylvatica* L. (Beyeler and Heyser 1997), the establishment of mycorrhizas resulted in reduced concentration of certain soluble and cell-wall-bound phenolics in the fine roots, which the authors suggested to be a prerequisite for ECM formation (Münzenberger et al. 1990, 1995; Beyeler and Heyser 1997). Accumulation of specific phenolic compounds in the mycorrhizal roots seems to be tissue-specific and dependent on the developmental phase of ECMs (Weiss et al. 1999), which may partly explain the inconsistent results.

The role of lignin in the regulation of ECM interaction is still unknown. However, as an agent increasing the strength of the cell wall (Plomion et al. 2001), it may participate in the control of fungal growth in the intercellular space of the epidermis and cortex as well as in the inhibition of the fungal penetration into the endodermis. Furthermore, as an organic compound, lignin modifies soil structure and nutrient composition and, thus, may affect the composition of the fungal community in the soil. In their study on the effects of CAD- and COMT-deficient transgenic *P. tremula* × *P. alba* on soil chemistry and biology, Pilate et al. (2002) found that neither N and C contents nor microbial biomass were changed due to the altered lignin structure

of the roots. In contrast, decomposition of the roots from transgenic trees accelerated. Saprophytic fungi are effective lignin decomposers, whereas ECM fungi have not been shown to have that ability (Cairney and Burke 1998; Cairney et al. 2003). However, these two important fungal groups of the soil are in close and partly competitive interaction, and the availability of the substrate to saprophytic fungi affects this interaction, which also refers to the nutrient availability of an ECM plant (Lindahl et al. 2002). Therefore, lignin may affect ECM association both directly in the root and indirectly via the conditions in the soil, and it is highly important to involve symbiotic ECM fungi in the studies performed with lignin modified trees.

To our knowledge, there are no published reports on the impact of lignin modification on the ECM interaction. However, there are three studies on the effects of altered phytohormone balance in the host tree on the formation of ECM symbiosis (Hampp et al. 1996; Kaldorf et al. 2002, 2004). Indole-acetic acid (IAA), which is known to regulate wood formation (Sundberg et al. 2000), has been suggested to play a crucial role in the development of mycorrhizal interaction (Barker and Tagu 2000). Both the in vitro study of Hampp et al. (1996) with *P. tremula* × *P. tremuloides* expressing *A. tumefaciensis* T-DNA auxin biosynthetic genes and the field studies of Kaldorf et al. (2002, 2004) with *P. tremula* × *P. tremuloides* expressing *rolC* gene showed that mycorrhiza formation was not disturbed by a modification of the host plant's phytohormone synthesis. The proposition of different ECM morphotypes in the roots was also nearly similar in both control and transgenic lines (Kaldorf et al. 2002, 2004). We have studied lignin biosynthesis in *B. pendula* and the effects of lignin modification on the interaction between *B. pendula* and an ECM fungus *Paxillus involutus* (Batsch) Fr. In our in vitro study in progress, both *B. pendula* lines transformed with the lignin biosynthesis precursor gene *COMT* and the control lines were able to form mycorrhizal symbioses with *P. involutus* characterized with both a fungal mantle and Hartig net (Fig. 5.2). However, the mycorrhiza frequencies differed among transgenic lines as well as between transgenic and control lines, but the differences could preferably be explained by other factors than lignin.

5.6 Conclusions

In the present paper we have put together the scarce amount of information that is available on the environmental aspects of lignin modified trees. At present, the scientific evidence on ecological interaction of lignin-modified trees with insect herbivores does not indicate any changes in preference or performance of insects related to altered quality or quantity of lignin. In the case of the interactions with ECM fungi, the variation found could also be explained by factors other than lignin. Furthermore, the question of whether these initial results scale up to a plantation level with diverse interactions

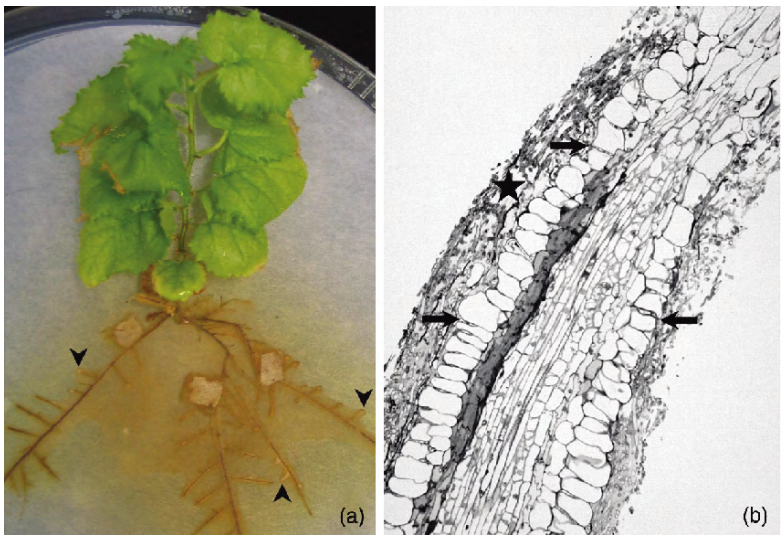

Fig. 5.2(a,b). Establishment of an ectomycorrhizal symbiosis between lignin-modified *Betula pendula* and *Paxillus involutus* in vitro: (a) roots of *B. pendula* inoculated with mycelial agar plugs of *P. involutus*. Fungal hyphae cover several root tips of the plant (*arrowheads*); (b) microscopic magnification (20×); fungal hyphae cover the root as a mantle (*star*) and penetrate between radially elongated epidermal cells (*arrows*)

remains unresolved. The great environmental and economical benefits that lignin modified trees might offer to the pulp and paper industry emphasize the importance of large field trials for multidisciplinary studies. The specific characteristics of forest trees, such as long rotation time, wind pollination and their key role in the ecosystems highlight the importance of environmental studies. The results of environmental studies and putative risks associated with genetic transformation technology have to be analyzed carefully. The risks associated with this technology should also be compared with the situation not to use genetic transformation technology (Walter 2004; Walter and Fenning 2004). Finally, the decisions for plantations with lignin modified trees are not only a scientific but also a societal issue. Therefore it is important that the political decisions are based on scientific evidence and proper risk assessment that keeps both context and perspectives in mind. Furthermore, it is highly important to ensure that the decisions attend to sustainable development.

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6 Modification of Cellulose in Wood

Matthias Fladung

6.1 Introduction

Modification of wood parameters is traditionally a major goal in forest breeding programs. The structure and composition of wood as a natural grown multilevel composite material has a strong effect on its mechanical properties. Moreover, wood material as a complex mixture of different chemical compounds (long polymer chains [cellulose] and amorphous substances [lignin, etc.]), all having different thermal and hygro-expansion properties, is subject to interaction phenomena that make its mechanical performance dependent on ambient conditions.

Wood consists of a number of different cell types (vessels, tracheids, parenchyma and sclerenchyma cells). The different tissues and cells are organized in different wall layers (P, S1, S2, and S3). Chemically, dry wood has about 40–50% of cellulose, 15–35% lignin, and 25–40% hemicelluloses (Nevell and Zeronian 1985). Composition of wood, however, is very dynamic; it can be influenced by developmental as well as environmental parameters.

Breeding and selection methods have been employed for genetic improvement of many plant species. In forest trees, however, these approaches may have limited application because of their long generation cycles. Forest trees are long-lived and have extended vegetative phase ranging from one to several decades. So far, no bred tree revealing significant changes in wood parameters has been obtained with exception of a naturally occurring Loblolly pine (*Pinus taeda*) mutant showing altered lignin composition (Ralph et al. 1997). This mutant reveals a complete block of the cinnamyl alcohol dehydrogenase (CAD) enzyme, which converts coniferaldehyde to coniferyl alcohol, the primary lignin precursor in pines (Ralph et al. 1997).

For enhancing ethanol fuel and fibre production a promising approach is to select and breed trees revealing high wood substance yields. However, due to the limitations related to breeding of trees and the lack of effective marker-assisted technologies, gene transfer (genetic engineering) is an attractive tool for modification of wood and other components (Dinus et al. 2001; Strauss et al. 2001; Campbell et al. 2003).

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Tree Transgenesis: Recent Developments

The wood modification on one hand may affect lignin, either decreasing its content or changing its composition (Li et al. 2003). On the other hand it may be related to changes of cellulose content or features of the cellulose fibre. Both strategies are interesting for the pulp and paper industry. For lignin, comprehensive analyses have already been made on the effects of genetic manipulation on lignification and vascular integrity (Chap. 5 of this volume; Whetten et al. 1998; Anterola and Lewis 2002). Significant progress has been made to reduce lignin content or to modify lignin composition via the inhibition of key enzymes in lignin biosynthesis (Li et al. 2003). However, cellulose content can also be affected when the activity of "lignin-enzymes" are suppressed or over-expressed.

Very little is known about strategies to modify cellulose content or fibre characteristics in trees in the available literature. Molecular genetics of cellulose biosynthesis in trees has already been summarized by Doblin et al. (2002) and Joshi (2004). In this review, I will mainly discuss modification of cellulose content or fibre length. However, because lignin and cellulose pathways are interconnected, some results regarding lignin modification are also included.

The consequences of the genetic manipulations of wood parameters with respect to fitness values of the trees or wood mechanical characteristics can be studied when the transgenic trees are tested under natural environmental conditions. In such investigations possible pleiotropic effects and other related potential risks to lignin or cellulose modifications may be studied. These field trials are important before introducing transgenic trees in for commercial wood improvement programs.

6.2 Modification of Lignin (and Cellulose) Content via "Lignin-enzymes"

For the understanding of genes and genomes of plants in general or developmental genetics in annual plants in particular, significant progress has been made in the last 10–15 years on genomics of model plant *Arabidopsis thaliana* to address developmental genetics and other plant biology questions. However, not much has been studied in *Arabidopsis,* which can be used as platform for genetic manipulations of economically important wood traits. Further, addressing some tree-specific biological problems, e.g. unravelling cell wall formation in the woody dicot stem (Mellerowicz et al. 2001), a tree species is a better choice than *A. thaliana*. The genus *Populus* has become a model for trees because of its relatively small genome, rapid growth, availbility of gene maps, and relatively easily amenable for genetic transformation (Taylor 2002; Wullschleger et al. 2002). Therefore, most of the research work studying lignin biosynthesis and its role in the wood-forming process has been made in *Populus* rather than in *Arabidopsis* (Boerjan et al. 2003).

Lignin is a very complex natural polymer with many random couplings and the exact chemical structure of lignin is still unknown. Lignin is formed by removal of water from sugars to create aromatic structures and the reactions are irreversible. Different lignin monomers have been identified, and the types and proportions of the lignin monomers depend on the source in nature. Lignin resists attack by most microorganisms, and anaerobic processes tend not to attack the aromatic rings at all. Aerobic breakdown of lignin is slow and may take long time periods. Lignin is nature's cement along with hemicellulose, exploiting the strength of cellulose while conferring flexibility.

A very impressive example of lignin modification has been presented by Hu et al. (1999) who used antisense-RNA technique to inhibit 4-coumarate:coenzyme A ligase (4CL) gene expression. Transgenic trees with lowered 4CL expression exhibited up to a 45% reduction of lignin, but with a surprising 15% increase in cellulose content. The results indicate that lignin and cellulose deposition are regulated in a compensatory fashion, which may be considered when altering contents of either lignin or cellulose, or both.

Lignin quantity and quality have been considered to be two major barriers to wood and pulp production (Li et al. 2003). Indeed, decreasing lignin content or altering its composition using one monolignol biosynthesis gene significantly changes pulping characteristics (Jouanin et al. 2000; Baucher et al. 2003; Huntley et al. 2003). The down-regulation of Caffeic acid *O*-methyltransferase (COMT) or cinnamyl-alcohol dehydrogenase (CAD) in hybrid poplar (*P. tremula* × *P. alba*) resulted in substantial reduction in lignin content or higher lignin extractability in wood (Baucher et al. 1996) together with an increase in craft pulping, but cellulose degree of polymerization was not different (Lapierre et al. 1999). Pulping characteristics were also tested in transgenic aspen carrying genes encoding both the 4CL gene in antisense, and additionally the coniferaldehyde 5-hydroxylase (Cald5H) in sense orientation. Transgenic trees expressing either the one or the other gene show the already known effects of lignin reduction and cellulose accumulation in the antisense 4CL (Hu et al. 1999), and S/G-ratio increases in the sense Cald5H plants as shown for caffeic acid *O*-methyltransferase (COMT; van Doorsselaere et al. 1995; Jouanin et al. 2000). In the double transgenic trees the measured features were independent but additive yielding in transgenic trees with up to 52% less lignin, a 64% higher S/G ratio, and 30% more cellulose (Li et al. 2003).

Although these results appear to be encouraging, cellulose increase by reducing lignin content probably is not the best option for several reasons. First, altering more than one wood characteristic may not fit in the frame of the tree breeding program. Second, reducing lignin in the stem of a tree may have influences on other traits because lignin is important for the stabilisation of the plant structure. Thus, the biological fitness of lignin-reduced transgenic trees may be altered when released to the environment (Casler et al. 2002) making them more prone to breaks or to pathogen attack

(Fink 1999), though there is no evidence available supporting this hypothesis. Following a field trial study over several years, Pilate et al. (2002) could show that the fitness value of lignin-reduced transgenic poplar trees was similar to that of non-transgenic trees.

6.3 Modification of Cellulose Content via "Cellulose Genes"

6.3.1 Cell Wall Formation and Cellulose Synthesis

The biopolymer cellulose is the most abundant naturally occurring organic substance, being found as the principal component of cell walls in higher plants where it provides the main structural feature. Cellulose occurs in almost pure form in cotton (98%), and in lower percentages in flax (80%) and wood (40–50%). It is very insoluble, but drastic chemical disintegration reveals that cellulose is a long chain polymer, made up of repeating units of glucose, a simple sugar. Light scattering methods reveal that the length of cellulose chains range from 2000 glucose residues in cotton up to 14,000 in secondary walls of wood, and there is no branching (Haigler and Brown1985).

The special properties of cellulose result from the association of the long chains to form fibres called microfibrils (Brown 1996). The microfibrils associate to form larger fibrils or fibres, which in the secondary walls are then laid down in a criss-cross pattern (http://www-biol.paisley.ac.uk/courses/stfun-mac/glossary/cellulose.html). The number of these glucan chains in each bundle varies from 36 in the elementary fibrils to more than 1200 in some algal species (Brown et al. 1996). In tree species the content of cellulose varies in the primary and secondary cell wall types. The elastic and expanding primary wall, containing largely pectins but also 2–15% cellulose and hemicelluloses, is 0.1–0.2 μm thick and cellulose microfibrils are irregularly oriented. In contrast, the secondary wall has orderly oriented cellulose microfibrils, is rich in cellulose (50–75%), and also contains lignin (20–30%) and hemicellulose (10–15%). It consists of three layers, namely S1, S2 and S3. While the outer and inner layers (S1 and S3, respectively) are relatively thin (less than 0.3 μm), the middle S2 layer is up to 5 μm thick.

Until 1996, when the first report on the identification of bacterial cellulose synthase gene homologs in cotton was published (Pear et al. 1996), only little information was available on molecular aspects of cellulose biosynthesis in plants. Later, it was found that a large multigene family of encoding cellulose synthase (*ces*) genes exists in *Arabidopsis* and other plants (Joshi 2004). This fact has initially lowered the inspiration to modify cellulose content or quality via a genetic engineering approach.

Specific plant cellulose synthases (*CesA*) are necessary for secondary wall synthesis in vascular tissues, which is critical to wood production. In *Arabidopsis* a family of at least ten CesA isoforms exists which by mutant

analyses have been shown to play distinct role/s in the cellulose synthesis or cell wall composition (Delmer 1999; Richmond and Somerville 2000; Joshi 2003; Joshi et al. 2004). The *CesA* mutants work indicates that three different CesA proteins interact as subunits within a cellulose synthase complex: CesA1, CesA3, and CesA6 form the complex in primary cell wall biosynthesis, whereas CesA4, CesA7, and CesA8 form the complex in secondary cell walls (Eckard 2003). Taylor et al. (2003) have shown that some CesA proteins interact with each other in the formation of cellulose synthesizing complexes which are located in the plasma membrane (Joshi 2004). The different cellulose content between primary and secondary cell walls may be attributed to different functional properties of individual CesA subunits. There is no evidence that other proteins interact directly with CesA subunits within the CesA complex, this though remains a possibility (Eckard 2003).

By direct screening of the monosaccharide composition of total cell wall hydrolysates, a number of different *Arabidopsis* mutants were isolated which show defects in cell wall composition (Reiter et al. 1997). These mutants, however, did not reveal significant changes in cellulose content. Other *Arabidopsis* mutants called irregular xylem (*irx*) are defective in secondary cell wall deposition (Turner and Somerville 1997). Examination of the cell walls of these mutants by using electron microscopy showed that cellulose content is decreased which resulted in an alteration of the spatial organization of cell wall material.

While immense progress has been made in the analyses of *Arabidopsis* cellulose biosynthesis pathway, it is, nevertheless, important to understand cellulose biosynthesis in trees with special emphasis to wood formation. Significant progress in cloning and isolation of *CesA* genes in a tree species has been obtained in the tree model *Populus*. A number of different *CesA* cDNAs have been isolated which show a very high similarity to respective *Arabidopsis CesA* genes (Wu et al. 2000; Joshi et al. 2004). Three of these *Populus* genes (*PtrCesA1*, *PtrCesA2*, *PtrCesA3*) are thought to be related to secondary cell wall *CesA*s mainly based on high amino acid similarity to the *Arabidopsis* secondary cell wall genes (Joshi et al. 2004).

To study whether cellulose content can be increased in plants by genetic engineering, it is first worthwhile to express the three *CesA* genes that make secondary cell wall cellulose in cells that do not normally have secondary wall thickening (Somerville, online 2004; Somerville et al. 2004). However, because it is likely to be deleterious to induce extra cellulose synthesis in cells that need to divide and expand to support normal growth and development, the genes must be placed under transcriptional control of a promoter that is active at a time that is compatible with normal development. This will allow production and propagation of the transgenic plants and will also facilitate studies of the consequences of induced expression of the *CesA* genes at specific time and places and to differencing degrees. The transgenic plants containing the ectopic *CesA* genes can be analysed for cell wall composition (e.g., cellulose and other polymers) and for effects on growth and

development. If increased cellulose is obtained from chemical induction of the genes, the next step will be to test the feasibility of engineering enhanced cellulose under the control of developmental stage-specific promoters.

6.3.2 Cellulose Degradation

Constitutive expression of poplar cellulase in *Arabidopsis* revealed severe effects on plant growth (Park et al. 2003). Cellulase over-expressing transgenic plants showed increased size of the cellulase synthase complex which is due to both larger leaf blades and petioles. This result is in agreement with the observation that suppression of cellulases achieved by either expression of the antisense gene or co-suppression of Cellulase mRNA by overexpression of the sense gene resulted in reduced leaf growth (Ohmiya et al. 2003).

Determination of the sizes of parenchyma cells (palisade and epidermal) in leaves of cellulase-over-expressing and control plants revealed that these cells were larger in leaves of the transgenic plants (Park et al. 2003). These changes were accompanied with changes in mechanical properties. Chemical analyses of cellulose composition in transgenic plants did not show significant differences to wildtype – only the amount of xyloglucan present in the 4% KOH-insoluble fraction was slightly higher (Park et al. 2003). Studies on microfibril structure using nuclear magnetic resonance (NMR) revealed that the transgenic plants have an increased proportion of *trans*-gauche conformation (one of the three preferred ones) at the C6 carbon of 1,4-β-glucan. The consequence of this modification is that the transgenic plants have a greater proportion of crystalline cellulose.

Similarly to the compensatory relationship of "reducing lignin leads to increase of cellulose", the reversal statements also seems be true. Tension wood is formed in response to mechanical stress or gravitational stimuli on the upper side of non-vertically growing stems. A widely known feature during the formation of tension wood is the increase of cellulose associated with reduced lignin content (Wu et al. 2000). The increased cellulose formation is caused by the unique role of cellulose synthase A in cellulose biosynthesis (Wu et al. 2000). This relationship clearly indicates the existence of complex but well-defined signalling mechanisms which regulates both cellulose and lignin biosynthesis.

6.4 Modification of Cellulose Fibre via "Hormone Genes"

Hormones are involved in regulation of many features during plant development like shoot growth and flower formation. Genetically transferring hormone or hormone-like genes for changing shoot growth may be an attractive starting point when aiming for modifying phenotypic and wood anatomical

features in trees (Table 6.1). Classically, modifications of shoot growth resulting in so-called dwarf or semi-dwarf mutants are very often caused by mutations in genes encoding proteins that regulate synthesis and/or signalling of gibberellin, a major plant hormone (for reviews: Hedden 1999; Olszewski et al. 2002; Sun and Gubler 2004; Fleet and Sun 2005). The gene mainly responsible for internode length is gibberellin acid20 (GA20) -oxidase, as identified in dwarf mutants of rice (Spielmeyer et al. 2002), potato (Carrera et al. 2000) or aspen (Eriksson and Moritz 2002). Other dwarf mutants like the brachytic2 (*br2*) in maize or the dwarf3 (*dw3*) in sorghum are defective of a protein responsible for auxin transport (Multani et al. 2003).

Induction of dwarfism has been achieved in tobacco by over-expressing gibberellin acid2 (GA2) -oxidase gene from *Arabidopsis* (Biemelt et al. 2004). In poplar, the same gene was found to be over-expressed in a dwarf transgenic hybrid poplar line obtained in a screening of several independently activation-tagged transgenic poplar lines in tissue culture, greenhouse, and field environments (Busov et al. 2003). Experiments on over-expression of the Arabidopsis GA3-oxidase gene in transgenic aspen clearly show that ectopic expression of this gene alone cannot increase the flux towards bioactive GAs; thus the 20-oxidation is the limiting step (Israelsson et al. 2004).

When the GA20-oxidase gene is over-expressed in transgenic poplar, elongated shoot growth (Fig. 6.1a) with increased biomass production was observed (Huang et al. 1998; Eriksson et al. 2000; Biemelt et al. 2004). These characteristics were positively correlated with rate of photosynthesis (Biemelt et al. 2004), early flowering and decreased seed dormancy (Huang et al. 1998), and small leaves (Fladung, unpublished). Detailed investigations on gene expression alterations in wood-forming tissue of GA20-oxidase over-expressing trees showed that highest transcript changes occurred in genes generally involved in the early stages of xylogenesis, e.g. cell division, early and late expansion (Israelsson et al. 2003).

Transgenic plants over-expressing GA20-oxidase reveal a faster growth (Fig. 6.1b) with increased biomass production (Eriksson et al. 2000). The xylem of these transgenic trees form fibres with up to an 8% increased length compared to untransformed wildtype. Over-expression of active GAs in plant tissues on the other hand also lead to pleiotropic or other deleterious effects. In sorghum, expression analysis of a GA20-oxidase gene in embryos revealed a possible role of this gene in the hormonal control of germination (Perez-Flores et al. 2003). In poplar, GA20-oxidase transgenic plants reveal a poor rooting capacity causing problems during the transfer of transgenic plants into soil (Eriksson et al. 2000). GA20-oxidase gene was also identified as a candidate gene involved in seed dormancy and vernalization processes (Oka et al. 2001).

Transfer of the cytokinin biosynthesis gene *ipt* into poplar resulted in transgenic plants with severe effects on plant phenotype (von Schwartzenberg et al. 1994). *Ipt*-transgenic poplar revealed reduced apical dominance, a dwarfed appearance and inability to rooting. When

Table 6.1. Overexpression of hormone and hormone-like genes and their effects in transgenic trees

Hormone and hormone-like genes	Tree species	Overall effects	Effects on wood features	References
Gibberellins				
GA20 oxidase	*Populus tremula* × *P. tremuloides*	Elongated internodes and biomass improvement	Longer cellulose fibres	Eriksson et al. (2000); Fladung, unpublished
GA2 oxidase	*Populus tremula* × *P. alba*	Dwarf plants	–	Busov et al. (2003)
GA3 oxidase	*Populus tremula* × *P tremuloides*	Nearly unaltered growth pattern	–	Israelsson et al. (2004)
Cytokinins				
Ipt	*Populus tremula* × *P. alba*	Bud formation in the absence of exogenous cytokinins; shoots unable to root	–	von Schwartzenberg et al. (1994)
Auxins				
IaaH, IaaM	*Populus tremula* × *P. tremuloides*	Reduced growth rate	Altered wood anatomical traits like vessel size and density	Tuominen et al. (1995)
IaaL	*Populus tremula* × *P. tremuloides*	Pending leaves	–	Fladung and Ahuja (1996)
Rol genes	*Betula pendula*	Bushy growth habit, smaller leaves	Shortened xylem fibres	Piispanen et al. (2003)
	Populus tremula	Breaking of stem apical dominance, higher cumulative stem length	–	Tzfira et al. (1999)
–	*Populus tremula* × *P. tremuloides*	Dwarf plants, smaller leaves, altered growth, precocious flushing	Atypical late wood formation, different pyrolysis products, lower average cellulose content, higher arabino-galactan content	Fladung et al. (1996, 1997); Nilsson et al. (1996); Grünwald et al. (2000); Puls et al. (2003); Meier et al. (2005)

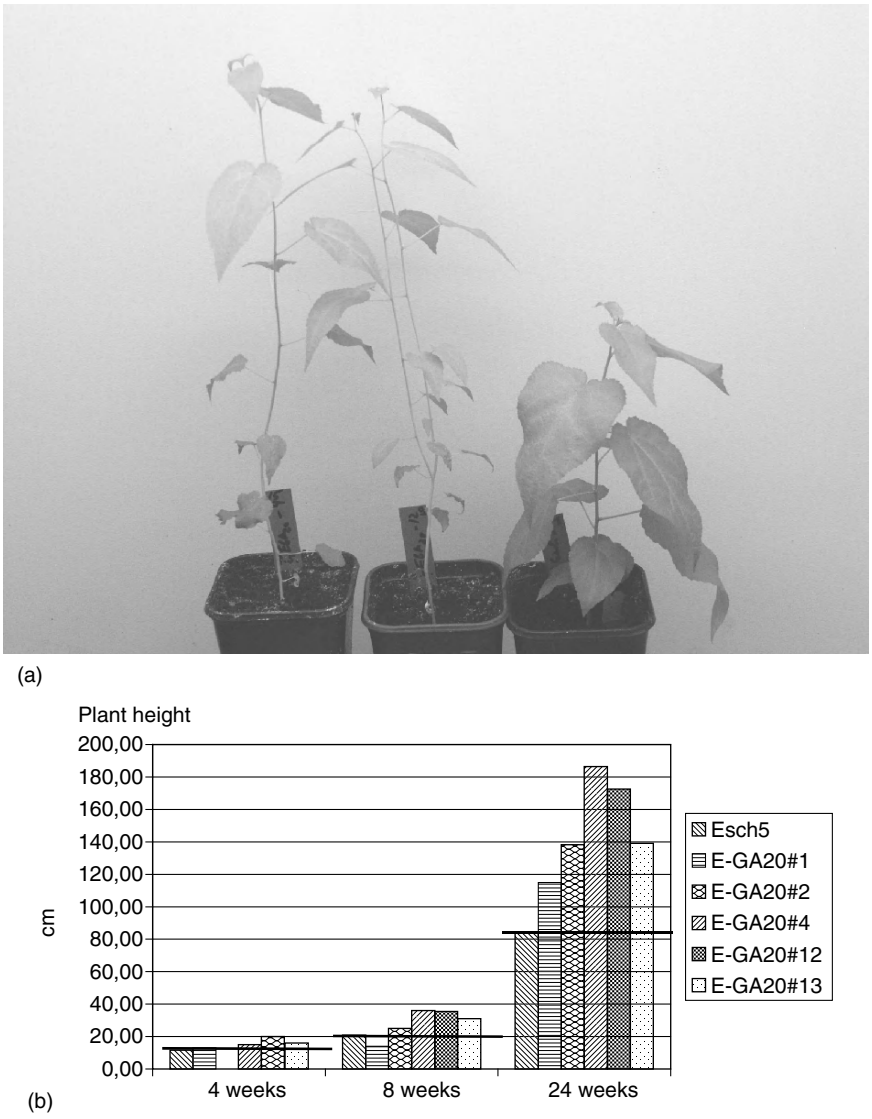

Fig. 6.1(a,b). Transgenic hybrid aspen over-expressing GA20-oxidase (*middle and left* plant, *right* plant is control) show: (a) an elongated shoot growth; (b) a faster growth (E-GA20: different independent transgenic lines; Esch5 is non-transgenic control)

Agrobacterium auxin biosynthesis genes *iaaM* and *iaaH* were used for transformation of *Populus*, transgenic plants showed increased levels of auxin in leaves and roots (Tuominen et al. 1995). Growth rate of the transgenic trees was reduced, and the wood anatomical traits like vessel size and vessel

density were altered. Severe phenotypic alterations were also observed in poplar transgenic to the *rolC* gene from *Agrobacterium rhizogenes* under control of the cauliflower-35S-virus promoter. Transgenic plants exhibited stunted growth with an increased number of small leaves (Fladung et al. 1996; Nilsson et al. 1996). *RolC*-transgenic poplar also revealed altered hormonal levels including gibberellin, auxin and cytokinins (Fladung et al. 1997). In spring, a precocious burst from bud rest was observed in 35S-*rolC* transgenic trees while initiation of cambial activity was not changed (Grünwald et al. 2000). Wood of 35S-*rolC* poplars revealed atypical late wood formation with thin-walled fibres. Characterisation of polymers in wood by analyzing their thermal degradation products using gas chromatograph and a mass spectrometer (Py-GC/MS) showed different patterns clearly discriminating wood of 35S-*rolC* poplars from control (Meier et al. 2003, 2005). Determining the cellulose, hemicelluloses and lignin contents in wood of 35S-*rolC* transgenic poplar revealed that the average cellulose content was 9.3% lower than the one of control trees (Puls et al. 2003). Interestingly the wood of the 35S-*rolC* transgenic poplar had a Klason lignin content, which was 4% higher compared to the non-transformed aspen trees. The wood of the transgenic 35S-*rolC* transgenic poplar also excelled by a higher arabinogalactan content. This hemicellulose component is typical for not fully differentiated wood tissues.

A second difference between the primary and secondary cell wall layers to the one mentioned above is the angle of microfibrils with respect to fibre axis. In the S1 and S3 layers the microfibril angle is 50–90° but 5–30° in S2 with lower angles in early wood than in late wood (Joshi 2004). Microfibril angle changes throughout the S2 cell wall layer. Within a fibre, the microfibril angle can change as much as 8° and among fibres within the same ring it can differ as much as 21° (Joshi 2004). Many fibres must be measured to obtain an accurate and precise assessment of microfibril angle. Microfibril angle is also a function of both height and rings from the pith. Microfibril angle is important for stiffness in wood with lower angles for enhanced wood quality. Hormonal treatments can be used to investigate the signal transduction pathways involved in regulation of cellulose microfibril angle (Jackson, online 2005). However, modification of the microfibril angle via hormonal treatments or even by using hormonal genes has not been tested so far.

6.5 Conclusions

Increasing cellulose content in wood of trees by altering the expression of genes involved in the lignin biosynthesis pathway seems not to be the optimal strategy because of possible undesired pleiotropic effects related to lignin reduction in transgenic trees. A possible approach to use cellulose biosynthesis genes has not, however, been tested so far. When aiming to

modify the length of cellulose fibres, a promising approach was followed by over-expression of the gene GA20-oxidase encoding for the final step in the gibberellic acid biosynthesis pathway. To avoid possible pleiotropic effects during plant development the GA20-oxidase should be controlled by an inducible promoter, e.g. stem-specific one.

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7 Heavy Metal Resistance and Phytoremediation with Transgenic Trees

Andreas D Peuke and Heinz Rennenberg

7.1 Introduction

Phytoremediation is the technology that uses plants to remove or degrade various pollutants from the environment. It has received significant scientific and commercial attention during the last decades (Salt et al. 1998; Gleba et al. 1999; Meagher 2000; Dietz and Schnoor 2001; Guerinot and Salt 2001; Krämer and Chardonnens 2001; van der Lelie et al. 2001; Schwitzguébel et al. 2002; Hannink et al. 2002; McGrath and Zhao 2003; Vassilev et al. 2004; Krämer 2005; Peuke and Rennenberg 2005a,b; Pilon-Smits 2005). Salt et al. (1998) and Dietz and Schnoor (2001) distinguish between different types of phytoremediation: (1) phytoextraction, (2) phytodegradation/-transformation, (3) rhizofiltration (removal of pollutants from aqueous phases by plant roots), (4) phytostabilization, (5) phytovolatilization (using plants to volatilise pollutants), and (6) removal of pollutants from the air by plants. Most attention is focussed on phytoextraction, phytodegradation and phytostabilization (Fig. 7.1).

For phytoextraction, plants are grow on contaminated soil and harvested from time to time while the biomass can be used in different ways depending on the type of contamination. As an example, plant material can be burned for energy gain (Fig. 7.1a). The aims are to remove pollutants from the soil and to concentrate them in biomass; final combustion of plant material will concentrate contamination further by a factor of around 10 in dry matter. The resulting ashes must be deposited in conventional dumps or added to a smelter. Recovery of metals from plant tissue ("phytomining"), which was done in the case of potassium ("potash") for centuries by humans, may be economical (Meagher 2000; van der Lelie et al. 2001). Phytomining may constitute a "green" alternative to existing, environmentally destructive, opencast mining practice or to exploitation of ore bodies which are uneconomic by conventional methods (Brooks et al. 1998). Plants are also able to take up radioisotopes like ^{134}Cs and ^{137}Cs which are of environmental concern after discharges from nuclear installations (White and Broadley 2000; Schwitzguébel et al. 2002).

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Plants can also transform pollutants taken up from the environment which is called phytodegradation (Fig. 7.1b). This is of particular significance for a range of harmful organics including most abundant environmental pollutants like polychlorinated phenols (PCB, e.g. dioxin), halogenated hydrocarbons (trichloroethylene: TCE) and ammunition wastes (nitroaromatics, e.g. TNT and GTN) (for phytoremediation of explosives see review of

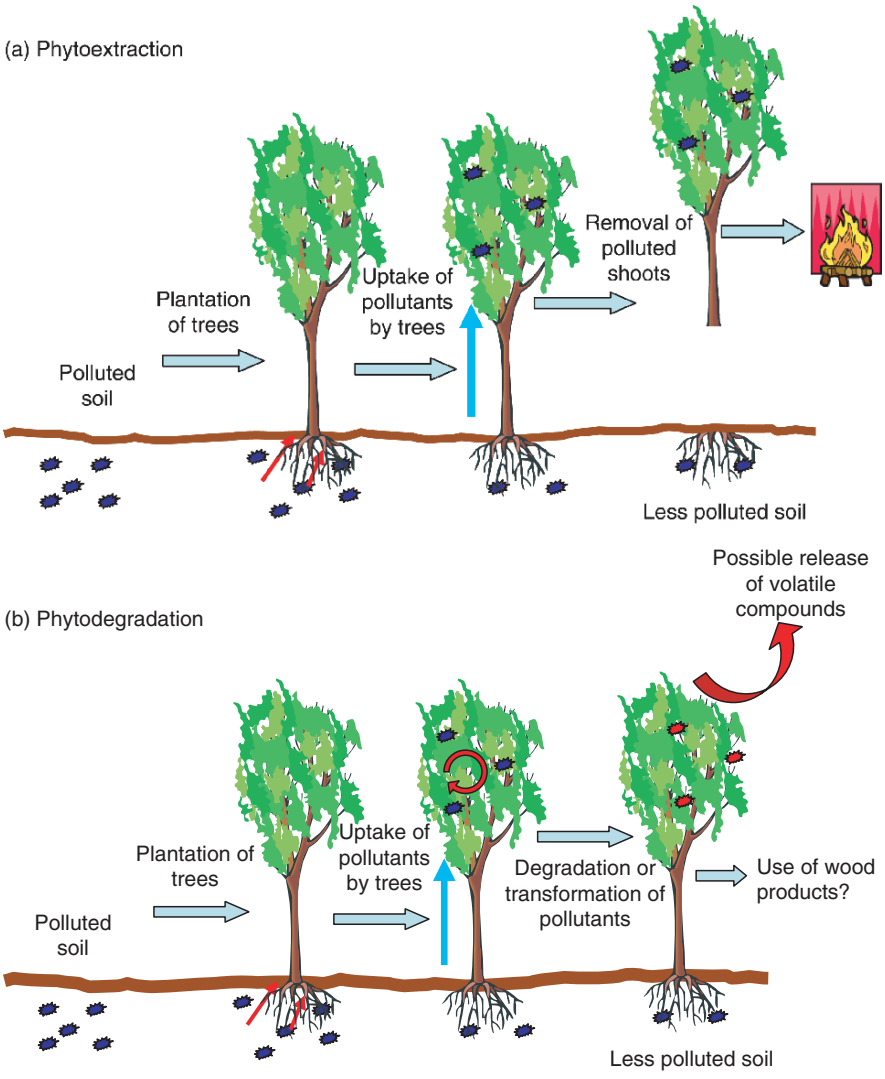


Fig. 7.1(a–c). Possible forms of phytoremediation: (a) phytoextraction; (b) phytodegradation; (c) phytostabilization

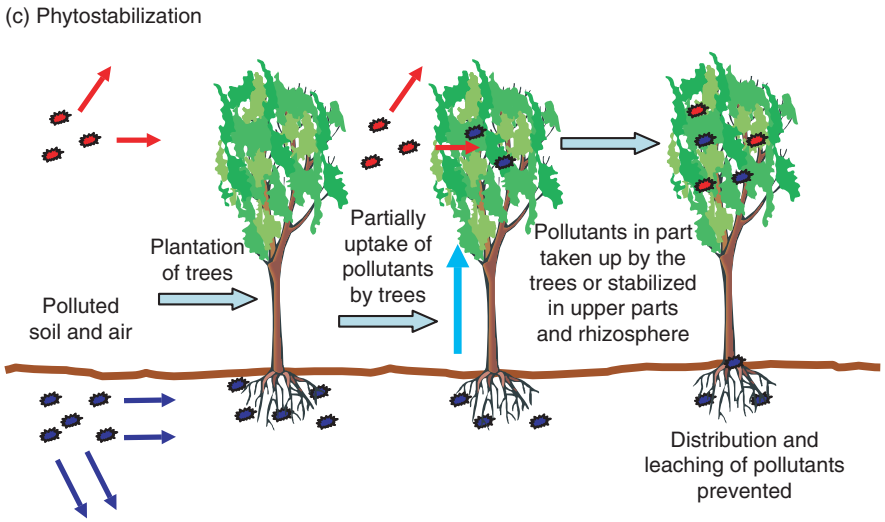


Fig. 7.1 (*Continued*)

Hannink et al. 2002). For organic pollutants the aim is to achieve its complete mineralisation to inorganic compounds (CO_2, H_2O, Cl_2) or less toxic compounds (Salt et al. 1998; Meagher 2000; Dietz and Schnoor 2001; Pilon-Smits 2005). The detoxification of organic pollutants in plants includes similar metabolic steps to that of heavy metals, like uptake and translocation, metabolisation (a. oxidation, reduction, or hydrolysis; b. conjugation with glucose, GSH, or amino acids) and sequestration into the vacuole. The best characterised transport system for toxic organics out of/into roots or vacuoles after conjugation with glutathione by glutathione *S*-transferases (GST) are ATP-binding cassette (ABC) transporters (Meagher 2000; Foyer et al. 2001; Dixon et al. 2002; Martinoia et al. 2002). Some elements like As, Hg, Fe, Se and Cr exist in a variety of cationic and oxyanionic species and thio- and organo-metallic forms. The transformation of toxic to less harmful species by plants is another approach of phytodegradation (Meagher 2000; Guerinot and Salt 2001).

Phytostabilization is the use of plants to keep contamination in situ and prevent wind and water erosion (Fig. 7.1c). In the simple case industrial plants or landfills are surrounded by a plantation of trees (e.g. poplars) to avoid contamination transport by wind. Cultivation of plants on a contaminated soil will also prevent erosion.

Compared to conventional methods of soil remediation the use of plants provide a number of advantages: (1) it is a low cost technology since, after planting, only low costs for field site management (weed control) and harvesting are necessary that are assumed to be ten times cheaper than

engineering based remediation (Vassilev et al. 2004; Pilon-Smits 2005); (2) it is "±CO_2-neutral" since during combustion the CO_2 released is not higher than the CO_2 fixed before by the plants during growth; (3) the product, biomass/wood can be used for several purposes including heat and energy gain. The drawback of phytoremediation is that it is a relative slow method, since it needs at least several years or decades up to centuries depending on the conditions or plants applied for reducing contamination (Schwitzguébel et al. 2002). During the time required for phytoremediation the area is characterised as contaminated, the plot is not available for business, sale or letting including loaning (SRU 2004). Additionally, after combustion the biomass ashes from phytoextraction of metal contaminated soils must be stored on special waste disposals.

Similar to a number of other new technologies, phytoremediation has to fight with public acceptability due to a lack of familiarity and predictability (Wolfe and Bjornstad 2002). This is p1articularly true if genetic modified plants (GMP) are involved (Schwitzguébel et al. 2002; Peuke and Rennenberg 2005b). Phytoremediation may become more acceptable at low-to-moderate amounts of contamination at shallow subsurface zone, which are assumed to constitute also low-to-moderate risk for human health and environment.

7.2 The Problem: Soil Contamination

Soil contamination can result in the damage of several soil functions and the contamination of surface water and groundwater. Next to consequences for ecosystems and other natural resources, the introduction of pollutants from contaminated areas into the human food chain via plant products or drinking water is of great concern (EU commission 2002; EEA 2003). The toxic effect of heavy metals in plants include generation of reactive oxygen species and free radicals, binding to S and/or N atoms of proteins and thereby leading to disruption and inhibition of activity as well as displacement of metal cofactors (Clemens 2001; Hall 2002; Pilon-Smits and Pilon 2002; Rea et al. 2004). After decades or even centuries of human activities in industry, mining, or military as well as farming and waste practice a huge amount of sites in developed countries shows high contamination with heavy metals or organic pollutants. The official report on the environmental situation in Germany (SRU 2004) mentioned in particular three main threats to the function of soils: (a) strains of area, (b) soil erosion and (c) input of pollutants. In the EU, an estimated 52 million hectares, representing more than 16% of the total land area, are affected by some kind of soil degradation. The largest and probably most heavily areas affected by contamination are concentrated around the most industrialised regions in north-west Europe, from Nord-Pas de Calais in France to the Rhein-Ruhr region in Germany,

across Belgium and the Netherlands and the south of the United Kingdom. Other areas where the probability of local soil contamination is high include the Saar region in Germany, the Po area in northern Italy, and the so-called Black Triangle region located at the corner of Poland, the Czech Republic and the Slovak Republic. However, contaminated areas exist around most major cities (EEA 2003). In Germany 362,689 potentially contaminated places are reported as to be suspected (SRU 2004). The estimates of the number of contaminated sites in the EU range from 300,000 to 1.5 million (EU commission 2002).

The costs for clean-up in the EU are estimated between €59 billion and €109 billion (EU commission 2002). The market for phytoremediation in USA is estimated to be actually $100–150 million per year which represents 0.5% of total remediation activities (Pilon-Smits 2005). The actual situation in Germany is that polluted soils from contaminated sites are only up to around 30% cleaned up in soil remediation facilities (SRU 2004). Most of the rest is stored on waste disposals underlining the necessity of research for alternative methods. Surprisingly, in the current report on the environmental situation in Germany (SRU 2004), the German term for remediation ("Phytosanierung") or a similar description cannot be found. Although in Europe commercial application of phytoremediation currently does not exist (Pilon-Smits 2005), it is expected to develop at the background of new members in the EU in East Europe and decreasing natural resources.

Bioavailability of a pollutant is more important than its concentration in the soil for the toxicity as well as for phytoremediation approaches (van der Lelie et al. 2001; Pilon-Smits 2005). Several factors have an impact on bioavailability, i.e. the chemical properties of the pollutant (hydrophobicity and volatility), the soil properties (particle size, organic matter content, redox conditions, pH), the environmental conditions (temperature, moisture) and the biological activities. In phytoremediation or phytostabilization projects bioavailability of pollutants can be manipulated by agronomic practices (Pilon-Smits and Pilon 2002). Plant density, species mixture and fertilization can enhance plant productivity. Simple watering will facilitate solubilization. Adding natural organic acids (malate or citrate) will have two effects: (1) lower the pH and (2) chelate metals. Adding lime increase pH and adding organic matter (humus or straw) will decrease the solubility of metals in the soil.

7.3 Some Specialists Can Deal with High Levels of Heavy Metals: Hyperaccumulators

A number of plant species (now up to 400–450 listed) are able to accumulate high amounts of heavy metals in their above-ground tissues (0.1–1%) on

metal-rich soils (Salt et al. 1998; Meagher 2000; Clemens 2001; Guerinot and Salt 2001; Hall 2002; Clemens et al. 2002; Pollard et al. 2002; Cobbett 2003; McGrath and Zhao 2003; Freeman et al. 2004). This was observed for essential nutrients (Cu, Fe, Zn, and Se) or non-essential metals (Cd, Hg, Pb, Al and As) which can cause toxicity already at low concentrations. The metal concentration in hyperaccumulators can be some orders of magnitudes higher than in not accumulating plants (100–1000-fold): 1% for Zn (up to 4%) and Mn; 0.1% for Co (up to 1.2%), Cu, Ni (up to 3.8%), As (up to 0.75%) and Se (up to 0.4%); and 100 ppm for Cd (up to 0.2%). Hyperaccumulators have an unusually high metal uptake and highly efficient chelation and compartmentation (Pilon-Smits and Pilon 2002; Pollard et al. 2002).

Although heavy metal accumulation is a rare phenomenon, it occurs widespread in different plant groups (Clemens 2001; Guerinot and Salt 2001; Broadley et al. 2001; Hall et al. 2001; Cobbett 2003; McGrath and Zhao 2003). Almost one-quarter of known hyperaccumulators are members of the Brassicaceae (Pollard et al. 2002; Freeman et al. 2004). The evolutionary selection pressure for hyperaccumulation may be protection against herbivory and pathogens (Pollard et al. 2002; Boyd 2004). Most hyperaccumulators are both small and slow growing and only few hyperaccumulating trees are known. A rare exception is *Sebertia acuminate*, a tree endemic to serpentine soils of New Caledonia (Guerinot and Salt 2001). After cutting, the stems of this tree are exuding latex which contains over 25% Ni (per dry weight) and the leaves contain up to 1.2% Ni in the dry matter. The background of the interest in plant metal hyperaccumulators is on one hand the benefit in human nutrition and on the other hand the understanding of detoxification mechanisms in plants, which can be used for phytoremediation (Gleba et al. 1999; Guerinot and Salt 2001; Pollard et al. 2002; Clemens et al. 2002; Cobbett 2003).

7.4 Dealing with High Concentration of Heavy Metals – Homeostasis, Tolerance, Detoxification

For most physiological and metabolic processes the maintenance and control of optimised metal concentration is necessary. For homeostasis as well as detoxification of supra-optimal metals concentration a number of cellular structures and physiological processes like accumulation in mycorrhiza, binding to the cell walls, exudation and a network of regulating metal transport, chelation, trafficking and sequestration are responsible in plants (Clemens 2001; Guerinot and Salt 2001; Clemens et al. 2002; Hall 2002).

Exposure of plants to heavy metals induces the synthesis of compounds that chelate these metals and, thus, contribute to their detoxification (Rauser 1999; Cobbett 2000; Clemens 2001; Hall 2002; Cobbett and Goldsbrough 2002; Rea et al. 2004). Among the chelators, sulphur-rich

peptides, i.e. gene-encoded metallothioneins (MT) and enzymatically synthesised phytochelatins (PC), are of particular importance, although the function of both groups of compounds is still under debate. The property of heavy metals to bind to thiol-groups of proteins, which is one of the toxic effects, will be exploited by these cysteine-rich polypeptides for detoxification. MTs are sulphur-rich proteins of 60–80 amino acids containing 9–16 cysteine residues and are found in plants as well as in animals and in some prokaryotes (Rauser 1999; Cobbett 2000; Cobbett and Goldsbrough 2002). PCs are a family of γ-glutamylcysteine oligopeptides with glycine or other amino acids as the C-terminal constituent. The γ-Glu-Cys units are repeated 2–11 times. The C-terminal amino acids of PCs include β-Ala, Cys, Ser, or Glu (Klapheck et al. 1994; Rauser 1999; Cobbett 2000; Cobbett and Goldsbrough 2002). PCs are synthesised from glutathione (GSH) and its derivates by phytochelatine synthase in the presence of heavy metal ions (Vatamaniuk et al. 1999; Ha et al. 1999; Cobbett 2000; Rea et al. 2004; Fig. 7.2b). The gene encoding phytochelatine synthase was cloned from *Arabidopsis* and yeast (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). Following Cd or Cu exposure, PCs were found in yeast, algae, lower and higher plants (Gekeler et al. 1989; Cobbett 2000). Cadmium is the most effective inducer of PCs, but Cu, Pb, Zn, Sb, Ag, Zn, or Hg also induce their formation (Cobbett 2000; Cobbett and Goldsbrough 2002; Schat et al. 2002; Rea et al. 2004). PCs form ligand complexes with these metals which are further sequestered into the vacuoles (Fig. 7.2b). Mutants in PC synthesis are hypersensitive to Cd and other metals (Howden et al. 1995). Using an inhibitor of GSH formation in hyper- and non-hyperaccumulating metallophytes showed that the PC-based sequestration is not essential for constitutive tolerance to heavy metals (Schat et al. 2002). Other low-molecular-weight organic acids (e.g. malate, citrate), amino acids (*O*-acetylserine, histidine) and nicotinamine (Cobbett 2000; Clemens 2001; Hall 2002; Krämer 2003) can also be used by plants as chelators in detoxification or transport.

A further mechanism of plants to deal with high metal concentration – similarly to mechanisms in halophytes/salinity – is the release of metals after uptake. Tobacco plants are able to exclude actively toxic Cd by forming and excreting Cd/Ca-containing crystals through the head cells of trichomes (Choi et al. 2001).

7.5 The Impact of Glutathione in Stress Resistance

Glutathione (GSH) occupies a central role in defence against oxidative stress, heavy metal pollution, and xenobiotics (Rennenberg and Brunold 1994; May et al. 1998; Foyer et al. 2001). Therefore enhanced biosynthesis and high levels of GSH and/or its metabolic precursor cysteine should improve stress resistance in plants. After reduction of sulphate the last steps of cysteine

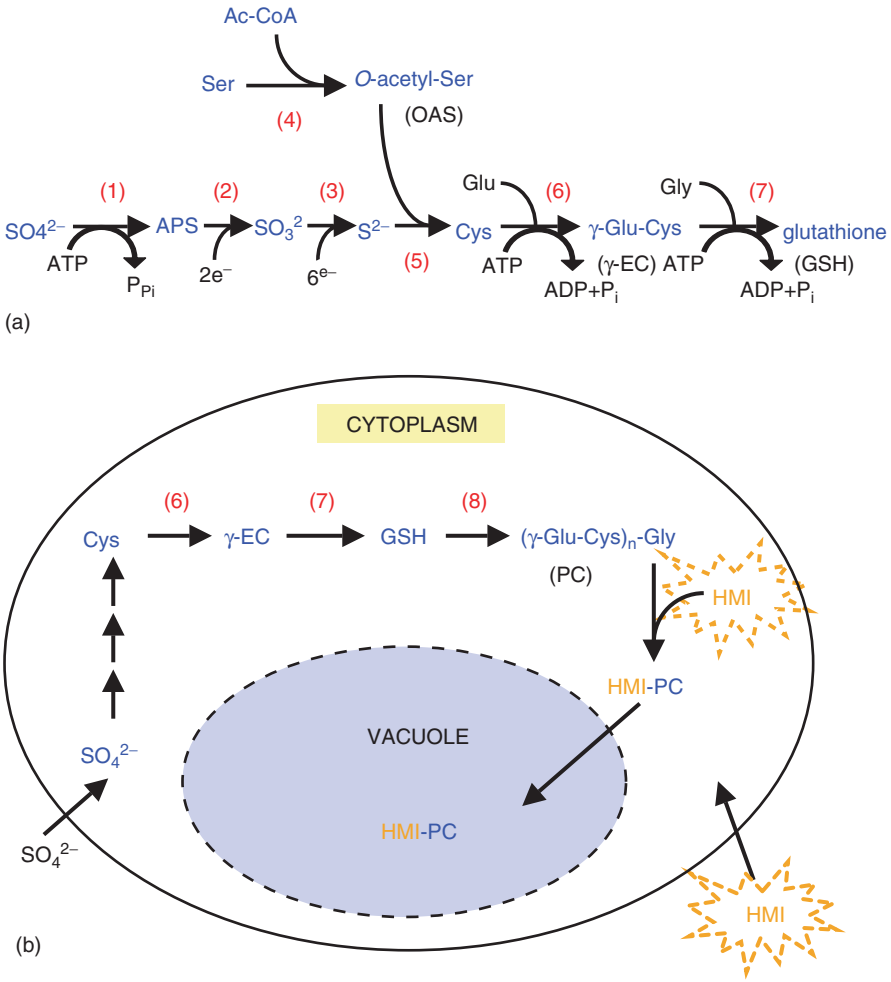
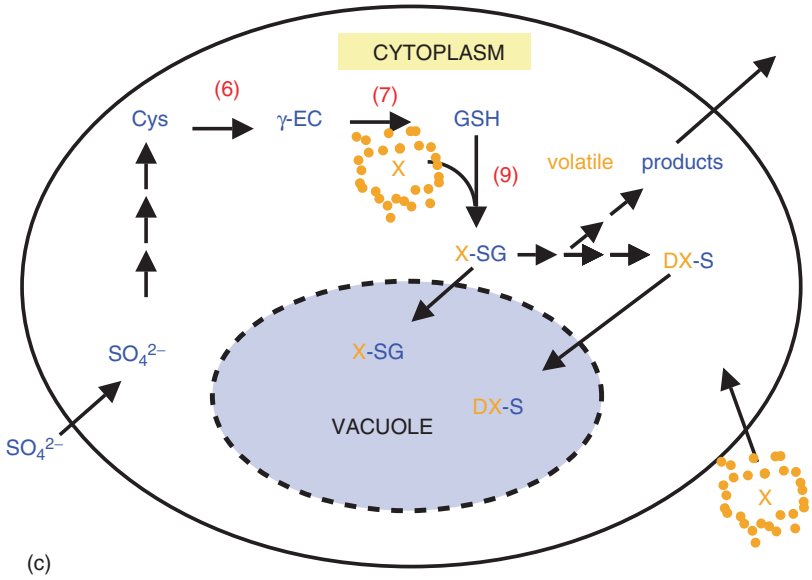


Fig. 7.2 (a–c). Role of glutathione in detoxification of heavy metals and xenobiotics: (a) reduction of sulphate and biosynthesis of cysteine and glutathione; (b) detoxification of heavy metal ions in plant cells by PCs and sequestration; (c) detoxification of organic pollutants by glutathione *S*-transferases (GSTs) and degradation of the reaction products including sequestration and possible volatilisation. APS: adenosine 5′-phosphosulphate, Ser: serine, Ac-CoA: acetyl-CoA, *O*-acetyl-serine (OAS), Cys: cysteine; γ-EC: γ-L-glutamyl-L-cysteine; GSH: glutathione; PC: phytochelatine; HMI: heavy metal ion; HMI-PC: heavy metal – phytochelatine complex; X: xenobiotics/organic pollutant; X-SG: xenobiotic-GSH conjugate; DX-S, product of X-SG degradation; (1) ATP-sulfurylase; (2) APS-reductase; (3) sulfite reductase; (4) serine acetyltransferase (SAT); (5) *O*-acetyl-serine(thiol) –lyase (OASTL); (6): γ-glutamylcysteine synthetase (γ-ECS); (7): glutathione synthetase; (8): phytochelatine synthase (PCS); (9): glutathione *S*-transferase (GST) (b and c modified from: Peuke and Rennenberg 2005b)



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biosynthesis are catalysed by serine acetyltransferase (SAT) and *O*-acetylserine (thiol)lyase (OASTL also called cysteine synthase CS) (Saito 2004; Sirko et al. 2004; Fig. 7.2a). These reactions can take place in the cytosol, the chloroplast or the mitochondria. GSH is synthesised in two ATP-dependent steps catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSHS) (Fig. 7.2a). Both enzymes are thought to be present in the chloroplasts and in the cytosol of leaf cells, and were also detected in roots (May et al. 1998; Noctor et al. 1998; Foyer et al. 2001). GSH synthesis is regulated by the availability of its constituent amino acids, by the control of transcription and activity of enzymes of glutathione biosynthesis and by hormonal control (Noctor et al. 1996, 1998, 1999; Foyer et al. 2001; Kopriva and Rennenberg 2004).

Due to the prominent role of GSH in stress defence, considerable efforts were taken to obtain plants with enhanced GSH levels or its precursors; such plants were expected to be more stress tolerant. Freeman et al. (2004) observed that the concentration of GSH as well as cysteine and *O*-acetyl-L-serine was strongly correlated with the ability to accumulate Ni in hyperaccumulating and non-accumulating *Thlaspi* species, both in natural habitats as well as under controlled conditions. This accumulation coincided with the constitutive activities of both SAT and glutathione reductase (GR).

Overexpressing SAT from *Thlaspi* in *Arabidopsis* increased resistance to Ni with respect to both growth inhibition and oxidative stress (Freeman

et al. 2004). Harada et al. (2001) transformed tobacco with a cytosolic cysteine synthase (CS) gene from rice. The transgenic tobacco exhibited a threefold higher activity of CS and higher PC levels upon Cd exposure and was tolerant to toxic levels of cadmium compared to the wildtype. Similarly in *Arabidopsis*, overexpressing *Atcys-3A*, the gene encoding cytosolic OASTL, led to an increase in GSH and PC levels but also to elevated Cd concentrations upon heavy metal treatment compared to the wild-type (Dominguez-Solis et al. 2001). Sirko et al. (2004) reviewed recent work on transgenic plants overexpressing SAT and/or OASTL and showed that not only the levels of first products, namely OAS and cysteine, but also GSH and other metabolites and enzymes activities increased. Additionally, in several cases the susceptibility to abiotic stress was lowered in the transformed plants. In *Brassica juncea* an increased capacity for GSH synthesis led to enhanced cadmium tolerance and accumulation (Zhu et al. 1999). This increased GSH synthesis capacity was achieved in *B. juncea* overexpressing of *gsh1* (coding for γ-ECS) from *E. coli*. This transformation resulted in higher concentrations of PCs, GSH, γ-EC and following Cd exposure, higher Cd levels in mature leaves.

In all cells where GSH is found, the reduced tripeptide form co-exists with the oxidized form (GSSG). To maintain the role of GSH in the redox status and defence against oxygen radicals and oxidants, GSSG formed can be reduced by glutathione reductase (GR) using NADPH as a reductant (May et al. 1998; Noctor et al. 1998). Transforming *B. juncea* by introducing a bacterial GR in the cytosol (cytGR) or the plastid (cpGR) was only successful with respect to enhanced Cd tolerance in the plastid transformation at the chloroplast level (Pilon-Smits et al. 2000). But the GSH level increased in the roots and this was assumed to be responsible for low Cd levels in the shoots after Cd treatment.

Overexpressing of PCS in *Arabidopsis* led to contradictory success in heavy metal tolerance: the plants became As tolerant, but Cd hypersensitive (Li et al. 2004). Due to the application of both heavy metals total thiol concentration increased, but the spectrum of thiols was very different. In any case Cd or As levels were increased in above ground tissue of the transgenic plants.

GSH and the soluble GST isoenzyme family play a crucial role in the degradation of several pesticides and defence of oxidative stress (Edwards et al. 2000; Dietz and Schnoor 2001; Dixon et al. 2002). GSTs are able to catalyse conjugation reactions between a number of xenobiotics (including pesticides) and GSH. The pesticide-GSH conjugates are generally much less toxic and more water-soluble than the original pesticide molecules and are sequestered into the vacuole (Edwards et al. 2000; Pascal et al. 2000; Foyer et al. 2001). In the vacuole GSH-conjugates can further be degraded (Edwards et al. 2000; Foyer et al. 2001). Therefore, GSH, GST and ABC-transporter play a prominent role in detoxification of heavy metals and organic pollutants in plant cells, besides other functions in metabolism cellular homeostasis,

transport, signalling, development and growth (Edwards et al. 2000; Foyer et al. 2001; Dixon et al. 2002; Martinoia et al. 2002).

7.6 Molecular Engineering to Improve the Performance of Plants in Phytoremediation

The effectiveness of plants for phytoextraction and phytodegradation of pollutants from soils depend on several physiological processes: (1) uptake by the roots, (2) loading into the xylem, (3) transport by mass flow in the xylem to the shoot in the transpiration stream, (4) uptake into leaf tissue,

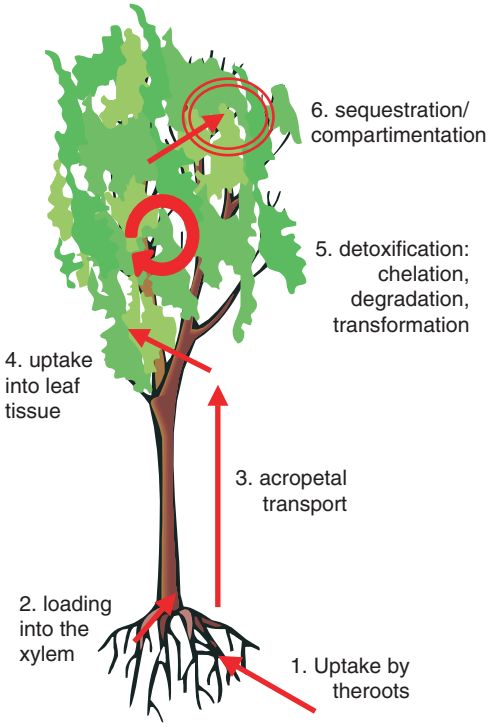

Fig. 7.3. Several physiological mechanism for resistance against pollutants and targets for bio-engineering and enhanced performance in phytoremediation. (1) uptake by the roots, (2) loading into the xylem, (3) transport by mass flow in the xylem to the shoot in the transpiration stream, (4) uptake into leaf tissue, (5) accumulation, storage and detoxification (chelation, degradation and transformation), and (6) sequestration/compartmentation of pollutants

(5) accumulation, storage and detoxification (chelation, degradation and transformation), and (6) sequestration/compartmentation of pollutants (Fig. 7.3) while maintaining metabolism, growth and biomass production (Gleba et al. 1999; Guerinot and Salt 2001; Clemens et al. 2002; Pilon-Smits and Pilon 2002; Pollard et al. 2002; Pilon-Smits 2005).

McGrath and Zhao (2003) showed that the efficiency of phytoextraction is determined by (1) biomass production and (2) the metal bioconcentration factor (ratio of metal concentration in the shoot tissue to the soil). With the exception of hyperaccumulators most plants possess metal bioconcentration factors less than 1 and cleaning up soils by half during a human generation is rather unlikely. To achieve a reduction of soil heavy metal contamination within one or two decades, it would be necessary to grow crops with a metal bioconcentration factor of 20 with a biomass production of 10 tonnes per hectare or a crop with metal bioconcentration factor of 10 with 20 tonnes per hectare biomass production.

Two strategies may improve the feasibility of phytoextraction of heavy metals: (1) to grow plants hyperaccumulating heavy metals in the harvestable above-ground parts, or (2) to produce high biomass with average heavy metal concentration in harvestable tissue within short time by fast growing plants. It would be desirable to combine both qualities of specialised plants to produce fast growing hyperaccumulators. This can be achieved by introducing transgenic plants for phytoremediation (Gleba et al. 1999; Meagher 2000; Dietz and Schnoor 2001; Guerinot and Salt 2001; Krämer and Chardonnens 2001; Pilon-Smits and Pilon 2002; Clemens et al. 2002; Krämer 2003; McGrath and Zhao 2003).

In contrast to growth of plants which depends on numerous factors in physiology (water and nutrient uptake, photosynthesis etc.), it is assumed that accumulation of heavy metals can be attributed to few gene loci (Clemens 2001; Pilon-Smits and Pilon 2002). The manipulation of PC production and/or GSH biosynthesis is a starting point to increase phytoremediation performance by molecular engineering (Noctor et al. 1998; Zhu et al. 1999; Cobbett 2000; Cobbett and Goldsbrough 2002). The genetic manipulation of several metal transporters in uptake and translocation has been shown to alter metal tolerance and accumulation (Pilon-Smits and Pilon 2002; Krämer 2005). The transformation of toxic forms of elements to less harmful species in plants (e.g. Hg(II) to Hg(0)) by transgenic enzymes is another approach of phytodegradation (Meagher 2000; Guerinot and Salt 2001; Pilon-Smits and Pilon 2002). A "second-generation" approach to engineer plants for phytoremediation is to overexpress vacuolar metal transporters. These GMPs would be able to detoxify heavy metals by pumping them into the vacuole while requiring only a small amount of transporters rather than a large amount of GSH, PCs or MTs (Tong et al. 2004). Additionally, plants can be genetically engineered to remove and/or degrade organic pollutants (Dietz and Schnoor 2001; Hannink et al. 2002). However, genetic transformation events will become negative for phytoremediation

purposes if they result in unchanged or even lower shoot concentration of metals (Pilon-Smits et al. 2000; Harada et al. 2001; Li et al. 2004; Krämer 2005) or lower growth also under polluted soils.

Up to now most transgenic approaches in phytoremediation have been tested only in laboratory experiments. Recently Pilon-Smits (2005) reviewed two field studies with transgenic plants. In one project Indian mustard overexpressing enzymes in sulphate/selenate reduction was tested on field sites polluted with Se, B, and other salts. Another field trial is testing transgenic yellow poplars for Hg volatilisation. In another ongoing field trial, transgenic poplars overexpressing γ-ECS are compared with wildtype plants for the removal of heavy metals in former copper mining regions (Peuke and Rennenberg 2005a,b and see below).

Transforming plants for phytoremediation may naturally result in higher resistance to pollutants. This may result in a higher fitness of these transgenic plants in contaminated ecosystems compared to the natural vegetation (Pilon-Smits and Pilon 2002; Pollard et al. 2002; Wolfe and Bjornstad 2002; Gressel and Al-Ahmad 2005). On the other hand, this will also be true for adapted or exotic plants. Therefore, before introducing specialised, exotic or transgenic plants into phytoremediation approaches a thorough risk assessment study should be performed in each case to prove the possibilities of ecosystem effects, the impact on biodiversity, or whether plants used for phytoremediation can become invasive.

7.7 The Use of Trees for Phytoremediation

Forest trees are exposed to a variety of stress situations, including extreme temperature, high light intensity, pathogen attacks, nutrient imbalances, etc. during their long lifetime. Plants possess several mechanism of stress defence ranging from morphological changes, to enhanced synthesis of defence compounds such as GSH, ascorbate, flavonoids, tannins, glucosinolates or alkaloids, and the induction of antioxidant enzymes, e.g., superoxide dismutases, ascorbate peroxidase, catalase, or glutathione reductase. These physiological reactions may lead to systemic resistance and hypersensitive reactions. Tree biotechnology is becoming an increasingly important tool for the remediation of contaminated environments (Vassilev et al. 2004; Krämer 2005; Peuke and Rennenberg 2005a). Fast growing trees like poplar species are good candidates for phytoremediation purposes due to their extensive root system, high water uptake due to high transpiration rates and, as a consequence, high acropetal transport with the transpiration stream, rapid growth, and large biomass production. Poplars can be grown in a wide range of climatic conditions. Additionally, poplar plantations can be used in "short rotation forestry" systems.

Trees are less expensive for phytoremediation than herbaceous plants since they provide the possibility of several cycles of decontamination

without the necessity to harvest whole plants and apply new ones every year. In addition, plantations of trees on contaminated soils can prevent erosion and the related spreading of the contaminants by wind (phytostabilization). After the first planting the costs for field management are relatively low and the products, biomass/wood, can be used at least for production of electricity and heat by burning in specialised wood power stations. Depending on the type and level of contamination the wood may be used for other commercial purposes. However, biomass from trees grown near mining sites and close to ore treated areas may exceed the limits of allowed values for various wood products (Rademacher 2005). On the other hand, it is very unlikely that materials from poplar will be introduced in the human food chain as well as in feedstuff for animals. Still mostly herbs were used in phytoremediation experiments (see, for example, Table 2 in Salt et al. 1998; van der Lelie et al. 2001; Schwitzguébel et al. 2002). Similarly, in bioengineering projects mostly herbaceous plants were selected as target organism for transformation (Krämer and Chardonnens 2001).

Populus spec. have shown naturally high tolerance to organic contamination. Poplars were effective in removing the herbicide atrazine and trichloroethylene from the soil (Burken and Schnoor 1996). Leaves of *Populus nigra* were highly tolerant against chloroacetanilide herbicides, obviously due to high GSH and GST levels (Kömives et al. 2003). From *Populus trichocarpa* × *deltoides* metallothionein genes (*PtdMTs*) were characterised and in heterologous expression the ability to confer Cd tolerance was demonstrated (Kohler et al. 2004). *PtdMTs* mRNAs were increased by Zn, but not by Cu and Cd. Karthikeyan et al. (2004) reviewed the potential of trees as non-target plants to remediate pesticide-contaminated soils. *Populus spec.* are able to transform and detoxify explosives (Hannink et al. 2002).

Overexpression of the bacterial mercuric reductase in *Liriodendron tulipifera* resulted in transgenic trees that were resistant to toxic levels of mercuric ions (Rugh et al. 1998). These trees are able to reduce highly toxic ionic Hg(II) to less toxic elementary mercury Hg(0) and to release elemental mercury tenfold more than wildtype. From a *Casuarina glauca* nodule a gene was recently isolated which coded for type 1 MT (*cgMT1*) and was most active in the root or old part of the shoot (Laplaze et al. 2002). In a project in Brazil, *Eucalyptus* species are in the process of being transformed with *cgMT1* (M. Quoirin, Federal University of Parana, Curitiba, Brazil, personal communication) and tested for phytoremediation purposes. In the case of success, this will allow to use also trees in tropical or sub-tropical regions for phytoremediation.

Poplar trees were transformed to overexpress γ-ECS from *E. coli* (Rennenberg 1997; Noctor and Foyer 1998). The transformed poplars contained higher levels of GSH and its precursor γ-EC than the wildtype (Noctor et al. 1996, 1998). Exposure of poplar plants overexpressing γ-ECS in the cytosol to 5 mmol/l Cd resulted in enhanced synthesis of PC and

accumulation of Cd in the plants (Rennenberg and Will 1999). Koprivova et al. (2002) studied the partitioning of Cd in different organs. Poplar lines overexpressing γ-ECS in the cytosol and the chloroplasts were treated with 2 mmol/l Cd in soil. Over a four-week period, the poplar plants were able to accumulate up to 5.3 mg Cd. Most remarkably, in young leaves of both transgenic lines, Cd was accumulated to concentrations 2.5–3 times higher than in the wild type. The increased allocation of cadmium to the young leaves represents a potential advantage for the phytoremediation process using the same plants over several vegetation periods. It was also shown that transgenic poplars tolerate more Zn than wildtype trees (Bittsánszky et al. 2005). Gullner et al. (2001) exposed wild-type poplar and two transgenic lines overexpressing γ-ECS to the chloroacetanilide herbicides acetochlor and metolachlor dispersed in the soil. The growth and the biomass of all poplar lines were markedly reduced by the two chloroacetanilide herbicides, but, this reduction was significantly smaller in the transgenic line than in wild-type plants. Herbicide exposures markedly increased the levels of γ-EC and GSH in leaves of each poplar line. The increase in the foliar amounts of these thiols was stronger in the transgenic lines than in the wild type, particularly in the upper leaves. Considerable GST activities were detected in leaves of all poplar plants. Exposure of poplars to chloroacetanilide herbicides resulted in a marked induction of GST activity in upper leaf positions.

7.8 Conclusions

In a current field trial transgenic poplars (*Populus tremula* × *P. alba*), which overexpress *gshI* from *E. coli* in the cytosol and, hence, possess enhanced GSH levels and an elevated capacity for PC production, are compared with wildtype plants for the removal of heavy metals at different levels of contamination and under different climatic conditions. The capacity of transgene poplar is being evaluated in field experiments in Germany (Saxonia Anhalt, district Mansfelder Land) and Russia (Middle Urals, Swerdlovsk oblast) in copper mining areas. The results will help to assess the capacity and biosafety risk of transgenic poplars for phytoremediation of soils. Although not yet finished, preliminary conclusions can be drawn from the first results of this field trial with transgenic poplar. The transgenic poplars were genetically stable and there are no indications to date of any impact on the environment. In the first measurements the transgenic trees have shown a higher capacity for accumulation of heavy metals, but only on the most highly contaminated soils. On control sites or sites with low contamination there were no differences in heavy metal concentration in the shoot between wildtype and transgenic trees (Peuke and Rennenberg 2005a,b). Thus, phytoremediation by trees seems to be a promising low cost technology.

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8 Transgenic Approaches to Engineer Nitrogen Metabolism

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8.1 Introduction

Plant growth depends on the availability of nitrogen. In fact, availability of inorganic nitrogen in natural soils is often a significant factor limiting plant growth and development. During the evolution of land plants a number of strictly regulated metabolic pathways evolved to guarantee the efficient acquisition, assimilation and economy of this essential nutrient. The uptake of inorganic nitrogen and its incorporation into amino acids has been a main area of interest in plant biochemistry and physiology for the last 30 years. Current knowledge on this topic has been recently revised and research efforts have centred on elucidation of key steps of the process, including nitrate uptake and reduction, incorporation of ammonium into amino acids (Lea et al. 2002). Basically, there are no differences in the way that herbaceous and woody plants acquire inorganic nitrogen from soil. With a few exceptions, most trees grow in places where nitrate or ammonium ions are the major forms of nitrogen available in soil. Therefore transporter systems for inorganic nitrogen acquisition and enzymatic complements for its reduction and assimilation could not differ very much from those described in herbaceous plants. However, the tree life cycle differs substantially from that of herbaceous plants, and the management of nitrogen reserves is also a relevant issue to be considered in plant growth and development. In this chapter we summarize available data for inorganic nitrogen uptake, assimilation and metabolism as well as the general strategies developed to engineer nitrogen metabolism in transgenic herbaceous and woody plants.

8.2 Nitrogen Uptake, Assimilation and Related Pathways

8.2.1 Nitrogen Uptake

Nitrate is the major inorganic nitrogen form in soil (Wolt and Wolt 1994). Its availability depends on several factors including both environmental (temperature, rain, etc.) and biotic (e.g. microorganisms) factors. Agriculture

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practices may affect the concentration and availability of nitrate in soil. Since nitrate cannot diffuse through biological membranes, plants have developed a robust system to adapt them to the wide range of nitrate concentration found in soils, a basic mechanism to assure their survival and adaptation to changes in nitrate availability.

The characterization of inorganic nitrogen transporters has been a hard task for biochemistry and molecular biology researchers, mainly due to the difficulties involved in the isolation of active plasma membrane proteins associated to the transport process. Uptake of nitrate or ammonium by root cells is in fact the result of a balance between the active influx and the passive efflux into soil. Thus physiological studies dealt first with the kinetics of nitrate transport into barley roots in different experimental conditions. These early studies allowed the identification of a transport system with a low value of K_m, the high-affinity transport system (HATS; Siddiqi et al. 1990; Crawford and Glass 1998), and the existence of inducible transport systems (Siddiqi et al. 1990). Physiological studies allowed the discrimination between different transport systems including inducible (IHATS) and constitutive (CHATS) HATS, and a low affinity transport system (LATS) that was constitutively expressed (Crawford and Glass 1998). Kinetic studies of ammonium transport also allowed the identification of both high- and low-affinity transport systems in rice roots (Wang et al. 1993a,b). In both cases, nitrate and ammonium are co-transported with protons into the root cells, and therefore, their transport is associated to the polarization of plasma membrane. However, transport of nitrate or ammonium to soil affects the pH in a different way since alkalinization is observed in plants grown in the presence of nitrate but acidification results when ammonium is present as the unique inorganic N form in soil (Grignon et al. 1997) (Fig. 8.1). Since acidification rapidly leads to the inhibition of transport in root cells, it also provokes the inhibition of plant growth.

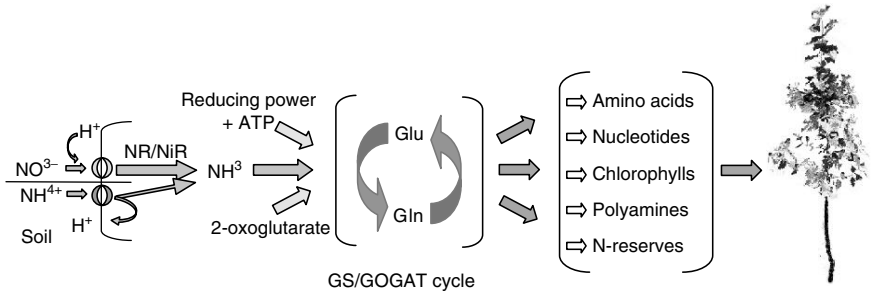

Fig. **8.1.** Assimilating nitrogen to build a tree. Inorganic nitrogen is incorporated into the root through specific transporters. Once nitrate has been reduced to ammonia by nitrate and nitrite reductase (NR/NiR), the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle is the main pathway for its transfer to the organic pool of nitrogen molecules. Glutamate and glutamine are the N-donors for the biosynthesis of all nitrogen compounds

The identification of genes involved in both nitrate and ammonium transport was initially achieved by the characterization of plants defective in nitrate assimilation, and by complementation of yeast mutants affected in the uptake of ammonium ions, respectively (Ninnemann et al. 1994; Huang et al. 1996). Although plants differ in the number of genes involved in the transport of nitrate and ammonium, gene families have been identified in different models as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* (Galván and Fernández 2001; Glass et al. 2002). The identification of genes involved in inorganic nitrogen transport systems in trees will require additional research efforts since limited information is still available about the physiology of the uptake process.

A main difference between herbaceous and woody plants is the fact that many tree species reduce the majority of nitrate taken in the roots. Thus, regulation of nitrate uptake in roots depends on several factors including the concentration of nitrate in soil, the C and N status of the plant, the accumulation of amino acids in the roots as results of both N assimilation and cycling, and the cycling pool of cytokinins that up-regulates the nitrate uptake at transcriptional level (revised in Gessler et al. 2004).

8.2.2 Nitrogen Assimilation

Since nitrate is not cycling through the plant in the phloem, it has to be reduced in both root and leaf photosynthetic cells. Monomeric nitrate (NR) and nitrite (NiR) reductases catalyse the reduction of nitrate to ammonium ion using respectively cytosolic (NAD(P)H) and plastidic (NADPH or reduced ferredoxin) reducing power (Fig. 8.1). Both enzymes have prosthetic groups acting as electron-chain transport in the catalytic mechanism. In NR, FAD, cytochrome b559, and a Mo cofactor are involved in the sequential transfer of electrons from NAD(P)H to nitrate; while a 4Fe-4S and a sirohemo group are involved in the transfer of electrons catalyzed by NiR. Different NR and NiR genes are found in plant genomes. Depending on the plant species, NR is encoded by 1–3 genes, while NiR is encoded by 1–2 genes. The short-term regulation of NR is well known since an inducible NR has been found in several plants and also NR gene expression is controlled by light and circadian rhythm. In addition, NR is regulated by phosphorylation and interaction with 14-3-3 proteins which modulate its activity in dark or under a carbon deficiency (Vincentz et al. 1993; Huber et al. 1996). NiR in photosynthetic cells is co-regulated with NR and specific light- and nitrate-response elements have been identified in the gene encoding ferredoxin-dependent NiR.

Ammonium ions produced by NR and NiR are incorporated to the organic pool of molecules in the reaction catalyzed by the enzyme glutamine synthetase (GS, EC 6.3.1.2). GS plays a central role in the complex matrix of plant nitrogen metabolism since the enzyme catalyses the ATP-dependent condensation of ammonium and glutamate to form glutamine, a precursor of

all nitrogen compounds required for growth (Fig. 8.1). Plant GS is a holoenzyme composed of eight subunits and exists as two major isoenzymes located in different subcellular compartments and displaying non-overlapping roles (Fig. 8.2). In the photosynthetic tissues of many angiosperms, GS2, a plastid-located isoform of GS, is responsible for the assimilation of ammonium derived from nitrate reduction and photorespiration (Ireland and Lea 1999). GS1, a cytosolic isoform, is the predominant enzyme in roots and non-photosynthetic tissues and less abundant in green tissues with the exception of C4 plants, that exhibit low photorespiratory activity (Ireland and Lea 1999). The biological role of GS1 is still not well defined, but it seems to be involved in the primary assimilation of ammonium from the soil and the recycling of ammonium released through metabolic processes other than photorespiration (i.e. phenylpropanoid biosynthesis or protein and chlorophyll breakdown). Since GS1 in the leaf is expressed specifically in the vascular bundles, a role for GS1 in transport of glutamine to other plant organs has been considered (Carvalho et al. 1992; Pereira et al. 1992). In addition, GS1 is expressed in photosynthetic cells of conifers, where GS2 is absent (García-Gutiérrez et al. 1998), and also in angiosperms during physiological differentiation of plastids (Gallardo et al. 1988; Gálvez et al. 1990) or under stressful situations such as water deficiency (Bauer et al. 1997) or pathogen attack (Pérez-García et al. 1998). Recent genetic and molecular approaches have shown that GS may be a key component of plant nitrogen use efficiency and yield (Hirel et al. 2001; Obara et al. 2001). All these recent reports suggest that cytosolic GS (GS1) plays a central and pivotal role in nitrogen metabolism that is essential for nitrogen-use-efficiency in higher plants.

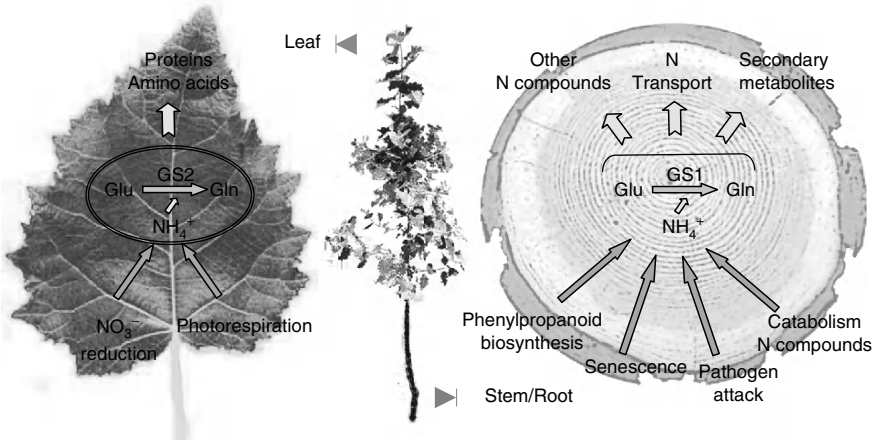

Fig. **8.2.** Plant GS isoenzymes have non-overlapping roles. In angiosperms GS2 is involved in the assimilation of ammonium from nitrate and photorespiration, while GS1 is responsible for assimilation of ammonium released in other secondary processes

The role of GS is associated to glutamate synthases (GOGATs) in the so-called GS-GOGAT cycle (Fig. 8.1). Thus, GS2 works with plastidic ferredoxin-dependent glutamate synthase (Fd-GOGAT, EC 1.4.7.1) in the production of glutamate, the precursor, with glutamine, of all plant nitrogen compounds in green tissues (Ireland and Lea 1999). GS1 may work with a plastidic NADH-GOGAT (EC 1.4.1.14) for the biosynthesis of glutamate in non-photosynthetic cells, especially in nodules of Fabaceae that are involved in the fixation of atmospheric dinitrogen. Plant genomes contain a small gene family of 2–5 members encoding GS1. In contrast, only 1–2 genes encode GS2, Fd- and NADH-GOGAT. Interestingly, genes encoding GS isoforms and GOGAT seem to be expressed in both angiosperms and gymnosperms with the exception of GS2. Thus, conifer GS is located only in the cytosol and no evidence for a plastid GS has been observed in biochemical, molecular and microscopy analysis (García-Gutiérrez et al. 1998). Therefore, GS1 is also involved in amino acid biosynthesis in conifer photosynthetic cells. In fact, two GS1 isoenzymes have been reported in pine, GS1a and GS1b with different expression pattern, molecular and kinetic properties (Ávila et al. 2001; de la Torre et al. 2002). While GS1b exhibits a expression pattern similar to angiosperm GS1, pine GS1a expression is associated to the development of chloroplast, suggesting that its physiological role may be similar to that of GS2 in angiosperms.

8.2.3 Carbon Flux for Amino Acid Biosynthesis

The assimilation of inorganic nitrogen into amino acid also requires the provision of carbon skeletons in the form of keto acids. Thus N and C demand needs to be adjusted by modifying the flux through respiratory ways to provide 2-oxoglutarate and other 2-oxoacids that are required for the biosynthesis of amino acids. 2-Oxoglutarate is provided by the reaction catalyzed by isocitrate dehydrogenase (IDH) (EC 1.1.1.41; 1.1.1.42). Although two different IDHs exist in plant cells, cytosolic $NADP^+$-IDH is the most active IDH enzyme in both angiosperms and gymnosperms, and it has been considered the main enzyme involved in the assimilation of ammonium and synthesis of glutamate when large quantities of the 2-oxoglutarate are required (Chen and Gadal 1990). The supply of 2-oxoglutarate through a cytosolic pathway involving aconitase and $NADP^+$-IDH is an alternative to the Krebs cycle enzymes for the provision of carbon skeletons for the assimilation of ammonium ions (Fig. 8.3). Regardless of its origin, the supply of 2-oxoglutarate for amino acid biosynthesis requires an increase in the flow of carbon metabolites through respiratory pathways to avoid the depletion of intermediates in the Krebs cycle. A requirement that must be met by increasing the flux via glycolysis, and increasing the production of phosphoenolpyruvate and its conversion into oxaloacetate by the carboxylation reaction catalysed by cytosolic phosphoenolpyruvate carboxylase (PEPC). The relevance of the

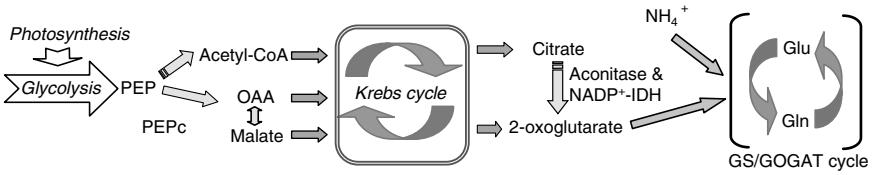

Fig. **8.3.** Provision of carbon skeletons for amino acid biosynthesis. 2-Oxoglutarate may be produced by cytosolic $NADP^+$-linked isocitrate dehydrogenase ($NADP^+$-IDH) or from Krebs cycle. Plant development requires increased flux through respiratory pathways to provide 2 oxoglutare for GS/GOGAT cycle

reaction catalyzed by PEPC is supported by reports describing the increase in PEPC activity during nitrogen assimilation in algae and higher plants (Valerberghe et al. 1990; Le Van Quay et al. 1991). Therefore, the incorporation of ammonium into amino acids requires the feeding of the Krebs cycle via the carboxylation of phosphoenolpyruvate. This flux is balanced during the cell life since surplus of malate and citrate can appropriately be stored into the vacuole and used through the Krebs cycle on demand for amino acid biosynthesis.

8.3 Relevance of N Metabolism in Trees

In angiosperm and gymnosperm trees, the study of nitrogen assimilation holds special interest. During the early stages of development GS expression is regulated somewhat differently in conifers than in angiosperms, specifically affecting the biogenesis of chloroplasts. In conifers, regulation of development of chloroplasts and expression of genes encoding chloroplast-located proteins are less dependent on light than in angiosperm species. This includes expression of genes implicated in ammonium assimilation (Cánovas et al. 1998; Suárez et al. 2002). The economy of reduced nitrogen has a special importance in trees since inorganic nitrogen is incorporated into amino acids in the source tissues and transported to sink organs through both xylem and phloem. The incorporation of amino acids into the storage sites is a relevant issue in deciduous trees. Thus, during fall the assimilated nitrogen is stocked as vegetative storage proteins (VSPs) in the trunk. Mobilization of these N-reserves occurs after dormancy to respond for the N-demand associated to formation of new leaves in spring (Wetzel et al. 2001). The coordinated allocation and cycling of amino acids requires the integration of environmental and N status signals at a whole-tree level. Since glutamine is a predominant amino acid in phloem, and regulates nitrate uptake in roots, it may be

considered as a signal molecule to integrate the N status of the plant. In fact, glutamine also regulates the C-demand for amino acid biosynthesis, and the accumulation of VSPs (Zhu and Coleman 2001). Therefore the modification of glutamine levels in engineered plants could have a relevant impact in tree development.

Moreover, woody tissues of the trunk of the trees are important sinks for the carbon and nitrogen assimilated during the tree life cycle. The phenylpropanoid pathway is responsible for the synthesis of a major group of structural and nonstructural constituents in vascular plants, such as lignins, lignans, flavonoids, suberins and tannins. The relevance of this pathway is not only qualitative but also quantitative, as 30–45% of plant organic matter is derived from the phenylpropanoid pathway. Therefore, vascular plants divert large amounts of carbon into the biosynthesis of this sort of products. The amino acid phenylalanine, and to a much lower extent tyrosine, are the only donors for the phenylpropane skeleton in this metabolic path-way. The enzyme phenylalanine ammonia-lyase (PAL) catalyzes the deamination of phenylalanine and tyrosine to cinnamic and *p*-coumaric acids, respectively. This is a crucial metabolic step connecting primary N metabolism through the shikimate pathway with the allocation of carbon for the biosynthesis of phenylpropanoids. In woody perennials, most metabolic flux through this pathway leads to the biosynthesis of lignin, an important constituent of wood. Nutrient use efficiency is one of the components of growth and therefore of high importance for wood production.

8.4 Genetic Manipulation of Nitrogen Metabolism

8.4.1 Studies in Model and Crop Plants

Transgenic plants could provide a means to study the role of various factors affecting and regulating inorganic nitrogen acquisition and assimilation. Consequently, there are many reports on this subject in the last years of the preceding decade (Table 8.1). Although the physiology of nitrogen uptake is well characterized (see Sect. 8.2.1), modification of nitrate uptake using transgenic approaches is complicated because of the existence of multiple genes involved in nitrogen transport systems in plants (Glass et al. 2002; Orsel et al. 2002). Consequently only a few studies have been reported (Liu et al. 1999; Fraisier et al. 2000). However, the metabolic pathways involved in the reduction of nitrate to ammonium and the assimilation of ammonium into amino acids are well understood in higher plants (Sect. 8.2.2). Early studies indicated that an increase in the level of key enzymes in transgenic plants resulted in very limited or no effect on the phenotype of the modified plant (Foyer and Ferrario 1994). This is particularly applicable to nitrate reduction because the overexpression of nitrate reductase and nitrite reductase affected

Table 8.1. Genetic manipulation of N uptake and assimilation in plants

Host species	Transgenes	Protein role	Promoter	Gene source	Reference
Arabidopsis. thaliana	*Nrt1.1*	Nitrate transporter	35S (CaMV)	*Arabidopsis*	Liu et al. (1999)
Nicotiana tabacum	*Nrt2.1*	Nitrate transporter	35S (CaMV) *rolD*	*Nicotiana plumbaginifolia*	Fraisier et al. (2000)
Solanum tuberosum	*NR*	Nitrate reductase	35S (CaMV)	*Nicotiana tabacum*	Djennane et al. (2002)
Arabidopsis, Nicotiana tabacum	*NiR*	Nitrite reductase	35S (CaMV)	*Nicotiana tabacum*	Crété et al. (1997)
Lotus corniculatus	*GS1*	Glutamine synthetase	35S (CaMV)	*Gycine max*	Vincent et al. (1997)
Populus tremula × *P. alba*	*GS1*	Glutamine synthetase	35S (CaMV)	*Pinus sylvestris*	Gallardo et al. (1999); Fu et al. (2003)
Nicotiana tabacum	*GS1*	Glutamine synthetase	35S (CaMV)	*Medicago sativa*	Fuentes et al. (2001)
Triticum aestivum	*GS1*	Glutamine synthetase	35S (CaMV)	*Phaseolus vulgaris*	Habash et al. (2001)
Nicotiana tabacum	*GS1*	Glutamine synthetase	35S (CaMV)	*Pisum sativum*	Oliveira et al. (2002)
Lotus japonicus	*GS1*	Glutamine synthetase	35S (CaMV)	*Medicago sativa*	Ortega et al. (2004)
Nicotiana tabacum	*GS2*	Glutamine synthetase	35S (CaMV)	*Oryza sativa*	Kozaki and Takeba (1996); Hoshida et al. (2000)
Nicotiana tabacum	*GS2*	Glutamine synthetase	Rubisco small subunit	*Nicotiana tabacum*	Migge et al. (2000)
Nicotiana tabacum	*Fd-GOGAT*	Glutamate synthase	35S (CaMV)	*Nicotiana tabacum*	Ferrario-Mery et al. (2000)
Solanum tuberosum	*ICDH*	$NADP^+$-isocitrate dehydrogenase	35S (CaMV)	*Solanum tuberosum*	Kruse et al. (1998)

N uptake but did not increased the yield or growth of plants (Crété et al. 1997; Djennane et al. 2002). In contrast, more recent studies have shown that genetic manipulation of genes involved in ammonium assimilation is a reasonable approach to enhance overall level of nitrogen assimilation and improved growth. Using transformation approaches, modification of expression of GS1 and GS2 has been achieved in a number of species including tobacco, *Lotus*, wheat and poplar (Table 8.1). Results obtained in the

molecular and physiological characterization of these plants confirm the relevance of GS isoenzymes in plant development, biomass production, and yield (Miflin and Habash 2002; Gallardo et al. 2003).

In comparison to GS, only a few studies addressed the modification of glutamate synthase and isocitrate dehydrogenase in transgenic plants by inhibition of gene expression (Kruse et al. 1998; Ferrario-Mery et al. 2000).

8.4.2 Production of Transgenic Trees and Consequences of Gene Manipulation

8.4.2.1 Enhanced Photosynthetic Metabolism and Vegetative Growth

Nitrogen assimilation and mobilization are crucial processes for growth and development of perennial species. Thus, modification of key enzymes in nitrogen metabolism is a reasonable strategy for enhancing growth of forest trees (Fig. 8.4). Increased levels of GS have been achieved in transgenic poplar by constitutive expression of a cytosolic pine GS1 under the direction of CaMV 35S promoter (Gallardo et al. 1999). The expression of the introduced pine GS gene has resulted in production of polypeptides of the correct molecular size which are assembled into a cytosolic holoenzyme in photosynthetic cells, a subcellular compartment where GS is not present in poplar (Fu et al. 2003) (Fig. 8.4). Interestingly, the enzymatic product of the transgene exhibited similar kinetic and functional properties (Fu et al. 2003). Although the role of cytosolic GS is not well defined in leaves of angiosperms, its expression in photosynthetic tissues has been associated with responses to biotic and abiotic stresses, fruit ripening, and leaf senescence (Gallardo et al. 1988; Bauer et al. 1997; Pérez-García et al. 1998; Brugière et al. 2000). Therefore,

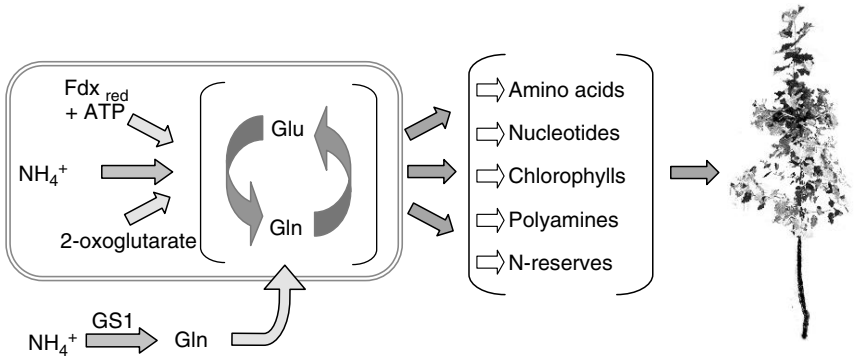

Fig. 8.4. Engineering a photosynthetic cell by the expression of cytosolic GS (GS1). Ectopic expression of a GS1 gene in mesophyll cells increases its capacity to assimilate ammonium. Enhanced biosynthesis of nitrogen compounds and plant development could be expected in the genetically engineered plant

modification of GS1 levels may have an important effect not only on nitrogen partitioning, but also on stress tolerance. There are several reports of transgenic plants with altered chloroplastic GS contents (Kozaki and Takeba 1996; Hoshida et al. 2000; Migge et al. 2000); however, the discussion in this chapter will focus on alteration of cytosolic GS expression.

Analysis of transgenic poplar lines expressing the pine cytosolic GS has revealed that ectopic expression of GS1 in the leaf leads not only to increased GS activity, but also to enhanced chlorophyll and protein contents (Gallardo et al. 1999; Fu et al. 2003). Moreover, greenhouse studies of transgenic poplars showed that enhanced GS activity in young leaves was correlated with increases in height growth suggesting that GS can be considered a marker of vegetative growth (Fu et al. 2003). In comparison with non-transgenic controls, transgenic trees exhibited a greater number of nodes and leaves as well as increased leaf area (Fig. 8.5). Similar results have been obtained in herbaceous plants (Fuentes et al. 2001; Miflin and Habash 2002; Oliveira et al. 2002), indicating that modification of cytosolic GS levels may be an appropriate approach for improving growth of crop species. The performance of transgenic trees expressing a pine GS transgene has also been studied in natural conditions (Fig. 8.5). Field trials of transgenic plants represent a relevant test to learn about the biological role in planta of introduced genes and proteins, and also to verify their potential use and interest for commercial applications.

In the particular case of trees, very limited information is available about seasonal variation in gene expression or how environmental conditions affects primary and secondary metabolism. A field study of eight independent transgenic lines and control plants has been carried out recently (Jing et al. 2004). The expression of the transgene was apparently stable throughout the period of study. Furthermore, transgenic hybrid poplars expressing cytosolic pine GS exhibited a higher vegetative growth than control plants during the three-year study. These data are in agreement to our previous results obtained from growth chamber and greenhouse studies (Gallardo et al. 1999; Fu et al. 2003). Transgenic poplars reached average heights that were 21, 36 and 41% greater than control plants after the first, second and third year of growth, respectively. Transgene expression affected plant features with time resulting in increased protein, total GS and glutamate synthase in leaves. However, neither differences in the large subunit of rubisco abundance nor water content were detected between lines suggesting that transgene expression did not alter the differentiation state of leaf. Moreover, no significant differences were found in total polysaccharide and lignin content in tree trunks. Whether or not GS1 transgene expression may alter the expression of other genes involved in primary and/or secondary metabolism is presently unknown. Further molecular characterization of the transgenic lines by transcriptomic and/or proteomic approaches (Bhalerao et al. 2003; Cánovas et al. 2004) is required to clarify this point.

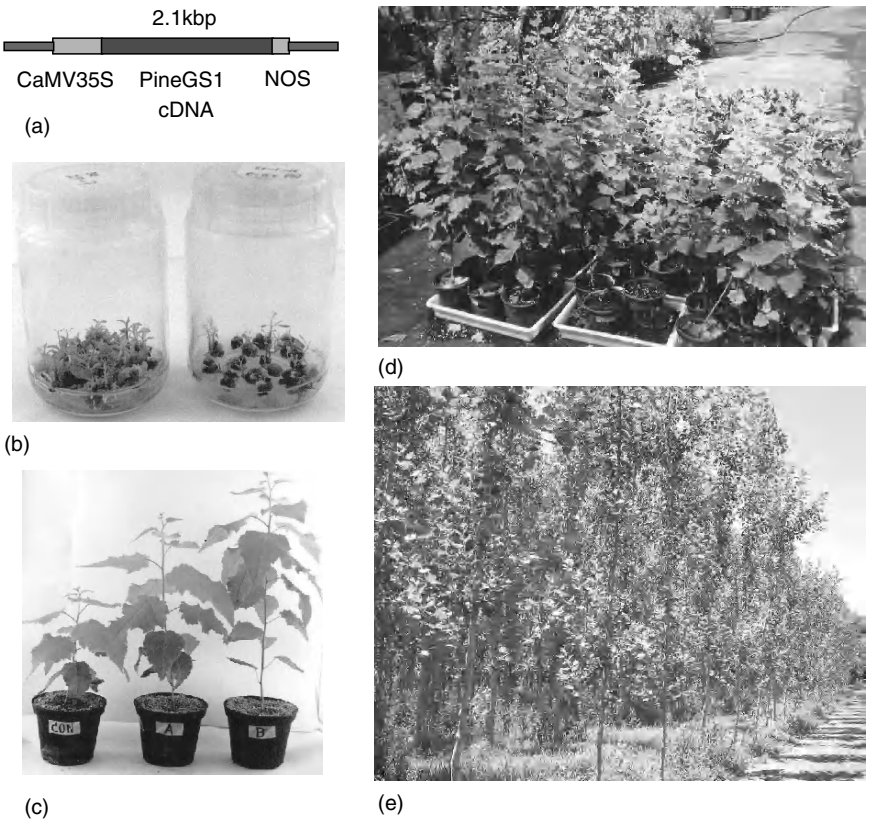

Fig. **8.5(a–e).** Steps in the production of transgenic trees overexpressing GS: (a) gene construct for the expression of pine GS1a (Gallardo et al. 1999); (b) in vitro clonal propagation of transformed hybrid poplar lines; (c) growth-chamber studies showing differences in the growth of control (*left*) and transgenic (*middle and right*) poplar lines (Fu et al. 2003); (d) greenhouse studies of transgenic trees; (e) field trial in Andalucía, Spain (Jing et al. 2004)

All the above findings support a strong role for GS1 in plant development and crop yield. Moreover, Vincent et al. (1997) reported that the overexpression of GS1 in *Lotus corniculatus* resulted in premature flowering and early senescence. This apparent acceleration of plant development could have application in accelerating flowering in plants with long juvenile periods, as in the case of woody perennials. However, if we consider that GS1 expression in transgenic angiosperms may accelerate the plant life cycle by enhancing vegetative growth resulting in premature flowering and senescence, why have angiosperms species evolved without GS1 expression in mature photosynthetic cells?. Phylogenetic analysis of nucleotide sequences suggests that plant GS2 evolved from a duplicated GS1 gene around the time of land plant evolution (Ávila-Sáez et al. 2000). Furthermore, the first multicellular plants evolved when oxygen levels in the atmosphere were similar to present day

levels. Transgenics overproducing GS1 have enhanced photosynthetic and photorespiratory capacities (Fuentes et al. 2001; Oliveira et al. 2002), a reported finding for enhanced expression of GS2 in transgenic tobacco (Kozaki and Takeba 1996). Taking all this into consideration, it is tempting to speculate that GS expression in photosynthetic cells was shifted from the cytosol (GS1) to the chloroplast (GS2) when land plants were already exposed to the present oxygen levels in the atmosphere, perhaps as an adaptive mechanism to overcome the high levels of ammonium released during photorespiration. Similarly, the product of a duplicated GS1 gene in *Drosophila melanogaster* was transferred from the cytosol to the mitochondria suggesting the adaptation of the mitochondrial isoenzyme to insect N metabolism inside the subcellular compartment (Caizzi et al. 1990). More data from other species transformed with GS genes will be necessary to get a better understanding of the biological relevance of cytosolic GS. Of particular interest for nitrogen metabolism in trees will be the molecular analysis of transgenic conifers overexpressing GS.

8.4.2.2 Increased N Use Efficiency

Cytosolic GS (GS1) has been recently proposed as a key component of nitrogen use efficiency in plants (Miflin and Habash 2002) and its metabolic role is particularly important for nitrogen remobilization and recycling in trees (Suárez et al. 2002; Cantón et al. 2005). Work carried out in our laboratory supports a higher capacity of transgenic trees not only in primary nitrogen assimilation but also in the reassimilation of ammonium released in different metabolic processes. This enhanced efficiency for nitrogen recycling could result in better exploitation of nutrient resources, improved efficiency of photosynthetic cells and faster plant growth. Fuentes et al. (2001) reported that higher performance of transgenic tobacco plants overexpressing GS1 was observed specifically when inorganic nitrogen availability was low. Similar results have been observed for GS-overexpressing poplars grown under low and high nitrate regimes (Man et al. 2005). When compared to non-transgenic controls, transgenic poplars lines displayed enhanced growth and the differences found were greater under low nitrogen. Furthermore, recent work using ^{15}N enrichment has shown that transgenic poplars have enhanced nitrogen assimilation efficiencies, particularly under conditions of low nitrate availability (Man et al. 2005). The impact of GS1 transgene expression on plant growth was previously observed in tobacco and poplar in conditions of low nitrogen nutrition or without the addition of nitrogen fertilizers (Gallardo et al. 1999; Fuentes et al. 2001; Fu et al. 2003). It should be noted, however, that the effect of pine GS1 transgene expression was relevant even under medium-high nitrate availability in soil, as observed in our growth studies carried out in the greenhouse and the field-grown trees (Jing et al. 2004). These findings strongly confirm that GS-overexpressing poplars have

increased nitrogen efficiency, better ability to exploit nitrogen resources in the soil and, thus, require lower nitrogen fertilization regimes. This could result in reduced risk of pollution, as is posed by current agricultural practices. Further research work is needed to improve our understanding of how nitrogen assimilation and nitrogen recycling influence nitrogen use efficiency in trees.

8.4.2.3 Increased Resistance to Stress

Transgenic tobacco and Arabidopsis plants overexpressing GS1 exhibited enhanced photosynthetic and photorespiration capacities (Fuentes et al. 2001; Oliveira et al. 2002). Transgenic poplars overexpressing pine GS1 also showed enhanced tolerance to water deficiency. Net photosynthetic rate was higher in transgenic trees before, during, and after recovery from transient water deficiency and there was evidence of increased photorespiratory activity (El-Khatib et al. 2004). These results suggest a greater resource allocation to photoprotective mechanisms. Interestingly, under conditions of severe drought stress transgenic plants maintained a higher relative abundance of GS1, GS2, glutamate synthase and rubisco as well as higher contents of chlorophyll and glycine than non-transgenic plants. Enhanced tolerance to phosphinothricine, a broad-spectrum herbicide that inactivates GS has also been observed in the transgenic trees (Pascual 2002) and this characteristic was associated to a higher content of GS and increased development in comparison with control-untransformed plants. All these reports provide evidence that enhanced GS expression is correlated with resistance to different types of stress.

8.4.2.4 Increased Nitrogen Reserves

The mechanisms whereby trees manage the reduced nitrogen during the onset of dormancy and resumption of active growth is of importance in developing a complete understanding of nitrogen homeostasis. VSPs serve as sinks for re-absorption of nitrogen from senescing leaves; thus they act as a reservoir of reduced nitrogen to support growth during the start of each growing season (Wetzel et al. 2001). Recent research has centered on the regulation of expression of VSPs and the effects of nitrogen status, photoperiod, and abiotic stress (Coleman 1997). Changes in growth in response to environmental factors may alter carbon and nitrogen partitioning, thus influencing VSP production (Zhu and Coleman 2001). During the growing period, the excess of nitrogen is accumulated as reserves in the cortex. The synthesis of VSP is subjected to seasonal changes and they accumulate mainly associated to the senescence of leaf during fall and winter (Wetzel et al. 2001). After winter dormancy, bud break and leaf development depends, in a first instance,

on the abundance of VSP accumulated in the cortex. Accumulation of VSPs appears to be a component of the overall nutrient use efficiency of the plant and, consequently, manipulation of VSP production could affect biomass production in trees.

A higher capacity to accumulate storage proteins in the cortex has been observed in the GS-overexpressing poplars growing in field conditions, which could be a consequence of their altered nitrogen partitioning, and contribute to enhanced vegetative growth following dormancy (Jing et al. 2004). When protein profiles of stem sections were investigated, it was observed the accumulation of a 32-kDa VSP concomitantly with the differentiation of the stem in poplar trees. This polypeptide may represent up 40% of total protein detected in the cortex during dormancy. Interestingly, it has been reported that expression of VSP in poplar is regulated by glutamine (Zhu and Coleman 2001) and this amino acid is the main form of nitrogen transported in poplar (Sauter and VanCleve 1994). The contribution of the cytosolic GS localized in the vascular bundles to nitrogen transport from source to sink tissues is important for tree nitrogen economy. In pine, GS1 is present in phloem cells of developing seedlings and xylem of adult trees (Ávila et al. 2000, 2001). Moreover, recent data from our group have shown that GS is specifically expressed in procambial cells during pine embryogenesis, suggesting a critical role for the enzyme in intercellular nitrogen transport even at early stages of plant development (Pérez-Rodríguez et al. 2006). According to these data, faster accumulation of the 32-kDa VSP in transgenic poplars could reflect the differences in the GS capacities detected in transgenic and control plants, and consequently a differential availability of organic nitrogen in the form of glutamine for tree growth and development (Jing et al. 2004). During the first year of our field study, and in agreement with previous findings (Fu et al. 2003), a higher GS content in leaf contributed to the higher growth of transgenics, and their higher content in protein and glutamate synthase could also affect their growth rate during the first year. Accumulated nitrogen reserves could explain the differences observed in growth at the end of the three-years study in the field.

Taking all these results together suggests that enhanced vegetative growth and development of agriculturally important species can be achieved by establishing lines with enhanced cytosolic GS expression in photosynthetic tissues. The co-localization of GS1 genes with QTLs for yield (Hirel et al. 2001; Obara et al. 2001) supports this strategy.

8.4.2.5 The Importance of C/N Balance

In plants, C/N balance is subjected to important fluctuations depending of a range of different factors such as photosynthetic activity, availability of nutrients or mobilisation of reserves and transport of carbon and nitrogen compounds from source to sink tissues. These changes will affect the fluxes

in major metabolic pathways with important consequences in plant growth. However, very little is known about the molecular cross-talk between primary and secondary metabolism in plants, and particularly in trees. In this sense, it has been reported that repression of lignin biosynthesis (by expressing an antisense 4CL gene) conferred growth advantages to transgenic poplars (Hu et al. 1999). Was this alteration disturbing the coordination between primary and secondary metabolism and resulting in growth differences? A number of recent reports obtained in herbaceous plants are supporting this possibility. For example, in transgenic tobacco expressing an antisense rubisco construct it was found that inhibition of photosynthesis and decrease in sugar levels leads to a general inhibition of nitrogen metabolism, and dramatic changes in the levels of secondary metabolites (Matt et al. 2002). Interestingly, the response was particularly clear in plants that received an excess of nitrogen. In a recent study, Rohde et al. (2004) have shown that disruption of PAL genes in *Arabidopsis* led to transcriptomic adaptation of components of the phenylpropanoid biosynthesis, carbohydrate metabolism, and amino acid metabolism, revealing complex interactions at the level of gene expression between pathways of primary and secondary metabolism (Rohde et al. 2004). All these data strongly suggest that fluxes in secondary metabolism are limited by the availability of precursors from primary metabolism. This implies that a coordinated regulation of primary and secondary metabolism is critical for plant growth.

A few studies have also examined the effects of nitrogen availability in the cross-talk between primary and secondary metabolism. Thus, Scheible et al. (2004) have found that nitrogen starvation led to coordinate repression of genes related to photosynthesis and plastid biosynthetic pathways whereas many genes for secondary metabolism were induced. In contrast, the supply of nitrate led to coordinated induction of amino acid and nucleotide biosyntheses and repression of the shikimate and phenylpropanoid pathways. It has also been reported that N availability alters the expression of genes that potentially impact wood properties in poplar (Cooke et al. 2003). Thus nitrogen fertilization experiments demonstrated that nitrogen availability is negatively correlated with lignin content in poplar (Cooke, personal communication) and maritime pine (M.J. Rodríguez-López, F.M. Cánovas, unpublished data) stems. In fact, during wood formation, the channelling of organic N towards lignin biosynthesis requires the control of nitrogen assimilation and transport of N-rich photosynthates such as asparagine, glutamine and arginine. In summary, increased evidence indicates that N availability affect the expression of genes assigned to pathways contributing to secondary cell wall synthesis such as lignin deposition. Furthermore, all these data indicate that a balance of C/N is critical in the regulation of metabolic fluxes.

How is the relative abundance of nitrogen and carbon compounds sensed in plant cells and N homeostasis maintained? Little is known about the molecular mechanisms involved in the regulation of C/N balance and how they influence plant growth and development. It has been shown that

2-oxoglutarate levels depend upon metabolic fluxes related to C and N metabolism and therefore 2-oxoglutarate levels can reflect C/N status. In bacteria, a protein named PII is able to interact with 2-oxoglutarate and regulate GS expression and enzyme activity, thereby coordinating C and N metabolisms (Little et al. 2000). A similar receptor protein for 2-oxoglutarate has also been characterized in plants (Hsieh et al. 1998; Cánovas et al. 2002; Smith et al. 2003; Sugiyama et al. 2004). In pine seedlings, the gene encoding PII-like protein is expressed in all organs tested but transcripts were predominant in non-photosynthetic tissues. In adult trees, transcripts were particularly abundant in developing xylem, suggesting a role for PII in the coordination of C/N metabolism during wood formation (F.M. Cánovas, C. Avila, F.R. Cantón, unpublished results). Further work is needed to confirm if 2-oxoglutarate could be a metabolic signal in a signalling pathway controlling gene expression and involving a PII sensing protein. In this context, the isolation and functional characterization of promoters for genes involved in nitrogen metabolism will be a critical step in the elucidation of common regulatory mechanisms for both C and N metabolism. Recent molecular and functional data showing the interaction a pine GS gene and MYB factors suggest a mechanism for a coordinated regulation of nitrogen metabolism and phenylpropanoid biosynthesis (Gómez-Maldonado et al. 2004). Furthermore, the expression of MYB genes was altered in *Arabidopsis* following changes in N nutrition (Scheible et al. 2004). These recent results suggest that MYB factors could be involved in the signalling mechanisms from molecular sensors of the C/N status and the target genes.

8.5 Conclusions

Natural forests cannot supply, in a sustainable way, the increasing world demands for wood. The production of plantation forests has the potential to meets these demands for wood in the near future if conveniently assisted by biotechnology to obtain increased yields and short rotation times (Fenning and Gershenzon 2002). Carbon emission from the burning of fossil fuels is one of the causes that influences current global warming. A way to increase the removal of carbon from the atmosphere is to maintain the natural forests and the sustainable increase of new tree plantations through intensive forestation programs. These new forests will have the added value of providing more biomass to sequester carbon dioxide from the atmosphere, thereby reducing the greenhouse effect.

As discussed above, the efficiency of nitrogen assimilation and partitioning is an essential component of plant growth and biomass production. Although recent progress has increased our understanding of how inorganic N is assimilated and metabolized in trees, further research is needed to explore the regulatory networks connecting N assimilation and recycling, tree growth and wood production.

A hallmark in this sense will be the integration of basic research and new advances in biotechnology to improve growth and development of economically important species. The intrinsic difficulties of woody plants as experimental models have limited the application of molecular techniques until recently. Nevertheless, the application of new functional genomic approaches such as large scale EST sequencing, gene expression profiling and proteomic approaches are allowing the identification of genes/proteins involved in several important processes in trees. Knowledge derived from these studies and the development of new molecular tools may allow increases in the productivity of forest trees in managed plantations through the rapid domestication of desirable traits (Campbell et al. 2003). These new developments, initially restricted to a few tree models (Lev-Yadun and Sederoff 2000; Taylor 2002), will be extended to other woody plants of commercial interest in the coming years. A relevant milestone has been the recent completion of the first complete genome sequence from a tree species. Due to the large genome size of conifers, poplar was proposed as the model tree to accomplish this goal. An international consortium has developed this project; the first draft was released in 2004 and is publicly available (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html; Brunner et al. 2004). The availability of the poplar genome information as well as the characterization of a large number of full-length cDNAs in conifer models will permit the functional characterization of proteins/enzymes encoded by the tree genomes, and the determination of their physiological roles. Thus, the expression, purification and molecular characterization of a large number of recombinantly expressed proteins are possible. DNA and protein arrays could now be generated and used as important tools for the functional characterization of woody plant systems. New adapted methods for the precise localization of mRNAs and proteins in tree cells and tissues are available and will provide new insights on how carbon and nitrogen metabolic pathways are organized in different cell types. Furthermore, as presented in several chapters of this book, functional studies in transgenic trees are feasible nowadays because routine transformation protocols via *Agrobacterium* are available for poplar and new advances have been reported in the last few years for conifers. This technology is providing new and valuable tools for tree functional genomics but is also allowing the test of genes potentially contributing to tree domestication (Strauss 2003). In tree species in which plant regeneration is a limiting, step such as conifers, somatic embryogenesis protocols offer the possibility of feasible regeneration of transformed plants (http://www2.vbsg.slu.se/sep/index.html; Keinonen-Mettälä et al. 1996; Ramarosandratana et al. 2001). The biological information derived from ongoing research efforts will greatly enhance our understanding of the molecular basis of tree structure and function. This new knowledge will have a clear impact on the future of forestry practices and management.

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Part C Biotic and Abiotic Resistances

9 Virus Resistance Breeding in Fruit Trees

MARGIT LAIMER

9.1 Introduction

Considering that five of the seven biblical species are fruit trees, their long standing economic importance and role as important element of food production becomes evident. Genetically, domestication of fruit trees involves a change in the reproductive biology of the plants by shifting from sexual reproduction to vegetative propagation (Zohary and Spiegel Roy 1975). As a rule, cultivated varieties of fruit trees are maintained vegetatively by cuttings, rootings of twigs, or suckers. This is in sharp contrast with the life cycle of their wild relatives, which reproduce from seed. Wild populations maintain themselves through sexual reproduction, are distinctly allogamous, assuring cross-pollination either by self-incompatibility or by dioecy, with high levels of heterozygosity.

In contrast to forest trees, fruit trees and grapevines have undergone thousands of years of domestication and centuries of breeding. Man-made selection led to some improved cultivars, sometimes at the cost of genetic variability (Zohary and Hopf 1993; Maghuly et al. 2005a). Most important fruit crops, in contrast to forest tree species, have been vegetatively propagated as cultivar clones, a factor which has also contributed to the spread of latent viruses and phytoplasmas (Laimer 2003a).

Modern cultivation of fruit trees further faces rapid rotation of orchards and a shorter life-span of individual plants. However, to release a new fruit tree cultivar it takes several years, sometimes even decades.

Given their nutritional and dietetic value, fruit crops contribute considerably to an improved world food production and human nutrition (Table 9.1) (FAOSTAT 2004). Current fruit production faces problems with biotic and abiotic stress factors during production, harvest and storage.

9.2 Importance of Viral Diseases

Accurate global figures for crop losses due to viruses are not available, but it is generally accepted that losses due to viruses are second only to fungi. There

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Table 9.1. Annual production in metric tons (FAOSTAT 2004) of the most important fruit trees world wide seriously affected by viral diseases

Host	Annual production 2004 in metric tons	Pathogens	Recorded losses
Citrus ssp.	108,094,504	CTV	45 million trees (Whiteside et al. 1988)
Vitis ssp.	65,486,235	GFLV/ArMV GVA/GVB	Bovey et al. 1980
Prunus ssp.	31,649,713	PPV PNRSV	Millions of trees (Kegler 1998)
Carica papaya	6,504,369	PRSV	Gonsalves et al. 2004
Theobroma cacao	3,302,441	CSSV	190 million trees (Ollennu et al. 1989)

exist several viruses affecting fruit trees, and although losses are difficult to quantify, Hull (2002) lists a few "famous" cases that have caused the death of millions of trees (Table 9.1).

Preventive measures, such as quarantine and the use of virus-free propagating stock, have been used to control some virus diseases, and tolerant or resistant cultivars are used to manage others. However, preventive steps alone do not adequately control vector-borne diseases, and adequate sources of host resistance or tolerance are not available for several important diseases (Garnsey et al. 2002).

The decision to engage in a resistance breeding programme employing biotechnological tools only makes sense in the case of pathogen-vector combinations that represent a serious threat to fruit production. This paper will focus on a few examples of biotechnological defence strategies developed against the most dangerous fruit tree pathogens, but it will also try to give an outlook to future developments.

9.2.1 Citrus Tristeza Virus

Citrus Tristeza Virus (CTV) (*Closteroviridae*) is the most devastating disease of *Citrus* worldwide. Millions of trees on sour orange rootstock have been killed or rendered unproductive by CTV-induced decline. Epidemics of tristeza decline began in the Western hemisphere in the 1930s and led to the loss of 30 million trees in Argentina, 10 million trees in Venezuela and 5 million trees in Brazil (Whiteside et al. 1988). CTV is transmitted semi-persistently by its specific aphid vector *Toxoptera citricidus* and also by other aphid species, which are less efficient vectors, but may be locally important in virus spread. CTV strains are

broadly grouped according to how they affect certain plants or scion/rootstock combinations, e.g. those causing mild symptoms, seedling yellows symptoms, decline on sour orange, stem pitting of grapefruit, and stem pitting of sweet orange (Lee and Rocha-Pena 1992).

9.2.2 Grapevine Viruses

Grapevine fanleaf virus (GFLV) (*Comoviridae*) is, together with *Arabis mosaic virus* (ArMV) and other Nepoviruses, the main causal agent of grapevine infectious degeneration (Bovey et al. 1980), one of the most damaging and widespread viral diseases affecting grapevine. GFLV causes either the rapid death of young plants or a progressive decline over several years. The virus is spread both via propagating material and nematode vectors.

The rugose wood complex of grapevine is found in most viticultural countries all over the world. The mealybug-transmitted Vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) are involved in the aetiology of Kober stem grooving and corky bark, respectively, two of the syndromes of the complex (Minafra et al. 1997). Again, no natural resistance to these viruses is known in *Vitis* sp.

9.2.3 Prunus Viruses

Plum pox virus (PPV) (*Potyviridae*) is classified by world plant quarantine agencies as the most important pathogen in apricots, plums and peaches (CABI/EPPO 1992). The virus is spread by aphids in a non persistent manner. "Sharka" disease results in heavy economic losses through a decrease in fruit yield and quality and in later infection stages in death of the infected trees and led to the destruction of millions of plum trees in Eastern Europe. After its appearance in South America (Herrera et al. 1998), PPV was encountered for the first time in 1999 in the US and Canada (Levy et al. 2000).

Stone fruits are also infected by *Prunus necrotic ringspot virus* (PNRSV) (Ilarvirus). Different strains of PNRSV exist (Mink et al. 1987), frequently in mixed infections with other viruses, e.g. with PDV, showing an increased severity of symptoms. PNRSV is spread by pollen and was considered the major stone fruit virus in North America.

9.2.4 Papaya Ringspot Virus

Papaya Ringspot Virus (PRSV) (*Potyviridae*) causes one of the most serious diseases limiting the economic viability and production of papayas and cucurbits in many areas throughout the tropics and subtropics including Hawaii, Taiwan, the Caribbean, Mexico, Brazil, Guam, the Philippines, and most

recently, Australia (Gonsalves 1994). PRSV is transmitted non-persistently by many species of aphids. PRSV strains are divided into two groups designated PRSV-p, infecting papaya, and PRSVw strains (formerly watermelon mosaic virus 1) found only on cucurbits.

9.2.5 Cacao Swollen Shoot Virus

Cacao Swollen Shoot Virus (CSSV) (*Caulimoviridae, genus Badnavirus*) causes one of the most devastating diseases of cacao in West Africa (Ghana, Nigeria, Togo and Ivory Coast). CSSV induces red vein-banding in young unhardened leaves, and shoot, stem and root swelling (Dale 1962; Adegbola 1971) and is transmitted by mealybugs. Yield reductions of up to 50% during the first year of infection, followed by the death of the cacao tree within three years may occur (Crowdy and Posnette 1947). Thresh (1991) reported that the disease is the most intractable and destructive to strike at the cacao industry in West Africa. In Nigeria, large areas of the best available cacao soils (69,638 ha) have been abandoned due to the devastation by CSSV in places referred to as 'areas of mass infection' (Thresh and Tinsley 1959).

9.3 Conventional Breeding Efforts for Virus Resistance in Trees

Host resistance probably is the most attractive approach to control of virus diseases in long-lived horticultural crops such as fruit trees, where short-term measures do not give any benefit It is worth mentioning that resistance, together with some agronomic control methods, is also the most sustainable means for virus control. However, the development of new cultivars is difficult and time consuming. The breeding cycle for fruit trees is usually ten years. Because most cultivars are highly heterozygous, the chances of combining virus resistance with all other essential horticultural features are low. Development of virus-resistant varieties using classical breeding has also been limited due to the scarcity of known resistance genes (Nemeth 1986), genetic barriers and the complexity of the target crops.

Although many types of resistance have been described, that can be effective against viruses (Fraser 1990) or their vectors (Khetarpal et al. 1998), unfortunately so far not so many have been found in fruit trees.

Plants are considered immune, when there is no evidence of virus infection. Trifoliate orange, for example, is often considered immune to CTV, although immunity in a strict sense at the cellular level has not been demonstrated (Garnsey et al. 1981). To take advantage of immunity in breeding programs the use of interspecific and even intergeneric crosses is required and sometime is not possible at all due to the lack of known resistance genes in related species.

In the case of qualitative resistance (Fraser 1990), genes responsible for the hypersensitivity (HR) or extreme resistance induce an absolute, strain specific reaction, which is determined in a mono- to oligogenic manner. Monogenic virus resistance may perform stably over years, but it might as well be overcome. Indeed, absolute resistance represents a considerable selection pressure on the pathogen population to produce new virulence genes. This fact deserves particular attention in the case of perennial tree crops, since the interaction time of host and pathogen are considerably longer than with any herbaceous plant. This is why quantitative or non-localising resistance has been pursued during the past decades as a more durable type of resistance, since it is broader, but only partial, not strain specific, and determined in a oligo- to polygenic manner. Again, with woody perennials some particular caution is required. Although tolerant plants may grow and yield satisfactorily despite sustaining a virus content that causes serious damage in sensitive cultivars, they are an important reservoir of inoculum and a significant problem in disease management. Some viruses cause different symptoms on different organs, e.g. PPV is characterised by leaf and fruit symptoms. There might exist significant correlation between strong leaf and weak fruit symptoms as in plum cultivar Anna Spaeth or none as in Stanley (Kegler et al. 1985). Indeed a critical reading of the literature on resistance in fruit trees suggests that there is no source of high level resistance able to protect against all virus strains, and cultivars resistant in one region or country may succumb to the same disease elsewhere.

Traditional breeding for finding or to introduce resistance to PPV started about the middle of the twentieth century at Cacak (Former Yugoslavia); in parallel, surveys of varieties resistant or at least less affected by PPV were undertaken, but little hope exists of finding a reassuring solution (Cociu et al. 1997; Hartmann 1998).

The qualitative localization resistance in the plum cultivar 'K4' (Kirke × Persikovaja) is strain specific (Kegler and Grüntzig 1992). The Hohenheim cultivar 'Jojo' and other descendants of the crosses Ortenauer × Stanley show quantitative hypersensitive resistance against PPV (Hartmann and Petruschke 2000), thus indicating a promising solution to plum production in Sharka infected regions (Hartmann 2002).

Some Middle-Eastern *Vitis vinifera* and *Vitis* species belonging to the subgenus *Muscadinia* show a good level of resistance to *Xiphinema index* and/or to GFLV, but this does not completely exclude viral infection (Walker and Meredith 1990; Staudt and Weischer 1992). Until now, traditional breeding methods have not allowed the release of new cultivars truly resistant to GFLV and/or its vector. The disease is controlled by soil disinfection using nematicides, but this procedure is forbidden in many countries due to high toxicity of the chemicals.

Most commercial cucurbits and nearly all papayas are susceptible to PRSV. Tolerance has been reported in a few papaya varieties and breeding lines (Conover et al. 1986), but these selections are not widely planted

because of relatively poor fruit quality. Genetic variation for resistance to PRSV is absent in domesticated papaya, motivating breeders to extend the search into a secondary gene pool. Many of the wild *Vasconcella* species, formerly members of the genus *Carica* (Badillo 2000) are intercompatible and can be cross-pollinated to produce hybrids with varying degrees of fertility (Aradhya et al. 1999). Hybrids between *C. papaya* and *V. quercifolia* produced in Australia and Hawaii have demonstrated PRSV-P resistance and some fertility (Drew et al. 1998).

In the case of CSSV, different control measures, such as eradication procedures (Thresh and Owusu 1986), cultural practices (Thresh et al. 1988), chemical control of the mealybug vector (Ollennu et al. 1989), cross protection using mild strains (Ollennu et al. 1996) and breeding for virus resistance (Legg and Lockwood 1981) have been applied to prevent further spread of the virus. However, none of these has been able to reduce significantly the disease incidence (Hoffmann et al. 1997). A considerable effort has been made over several years to select resistant or tolerant cacao genotypes already grown in Ghana or from subsequent introductions. However, the resistance obtained (Legg and Lockwood 1981) was insufficient to prevent virus spread under the prevailing conditions of high inoculum pressure.

9.4 Classical Cross Protection

"Cross protection" was originally described as the phenomenon of protecting a plant against the invasion of a severe disease causing strain by prior inoculation with an attenuated virus strain (McKinney 1929). Classical cross protection has been used to control virus diseases of a few crops (Fulton 1986). There are a number of reasons for the limited use and application of classical protection. First, the fact that a infective virus is used to deliberately infect plants makes researchers and growers reluctant to use this approach on a practical scale. Second, it is generally difficult to obtain mild strains that are of practical value. Third, there is some possibility that the mild strain might mutate and cause even greater problems than the control strain itself. Fourth, the mild virus strain may anyway cause serious damage in mixed infections with other pathogens. The inability to predict or control all potential hazards has precluded the preventive use of cross protection (Garnsey et al. 2002).

The control of CTV strains causing stem pitting on sweet orange and grapefruit in Brazil is the most successful example of cross protection to control a plant virus disease (Costa and Muller 1980). After the introduction of CTV to Brazil in the 1920s, by 1980, over 8 million trees of Pera sweet orange were cross protected and cross protection is still practiced in Brazil (Lee and Rocha-Pena 1992). Stem pitting induced by CTV reduced yield and, more importantly, fruit size of grapefruit in New South Wales (Australia).

Experiments conducted at two locations over a 21–25 year period showed the beneficial effects of cross protection as well as the effects of the climate (Broadbent et al. 1991).

Detailed studies over several years proved that an avirulent strain gave excellent cross protection against the virulent strain (Howell and Mink 1988). Although cross protection might be effective in controlling natural spread of the virulent strain, practical concerns with its use have been raised, some of which include mild isolate mutation, plant vigour reduction (Milbrath 1957), and introduction of other viruses into the orchard. The technique could be most appropriately be applied as a last resort in those orchards plagued by PNRSV as a method to contain and eliminate active Cherry Rugose Mosaic disease (CRM) areas (Howell and Mink 1988).

Selection of mild PRSV strains gave good protection in papaya against the severe Hawaiian strain. By 1991, more than 1700 hectares of cross protected orchards had been planted. A major concern is maintaining the purity of the mild PRSV strain and producing it under conditions which minimize contamination by severe strains. The specificity and limited availability of mild strains limits the widespread use of cross protection with PRSV. In Thailand, by contrast, the mild PRSV strain HA 5-1 did not provide a useful level of disease control (Yeh and Gonsalves 1994).

The use of mild strains of CCSV for cross-protection against virulent strains has long been known in Ghana (Posnette and Todd 1955; Ollennu et al. 1996). However, the method is limited as a means of control, because of the perceived risk that the virus, mild in cocoa, may damage other crops, and also the possibility of mutation into virulent strains if widely disseminated on million of plants.

9.5 Pathogen Derived Resistance (PDR)

Engineered protection offers a new approach to manage virus diseases, allowing the opening of completely new avenues of protection. Increased knowledge of both the molecular genetics of plant viruses and, more recently, also of their hosts' natural defence systems have resulted in the development of a number of novel ways to control virus diseases in plants.

Strategies for genetic engineering of resistance to virus are based on three types of transgenes: (a) pathogen derived transgenes, (b) plant derived transgenes including PR and R-genes and (c) non-plant, non-pathogen derived transgenes, e.g. antibodies and antiviral proteins (Sanford and Johnston 1985; Schillberg et al. 2001; Goldbach et al. 2003).

The use of plant derived transgenes allowing the introduction of natural R genes from one plant species to another has obvious advantages, since in the public perception it is more readily accepted than moving genes from other organisms. However, difficulties in the identification and characterisation of such genes

have retarded the development of this approach. Work towards the isolation of candidate genes from relatives of *Prunus* are in progress (Decroocq et al. 2005).

9.5.1 Transformation, Selection and Regeneration Approaches

Breeding of perennial fruit trees by traditional means requires long time periods due to the long generation time, the high degree of heterozygozity and multiple backcrosses needed to eliminate undesired traits. Conventional breeding by crossing is limited by reduced availability of suitable resistance genes in the gene pool. In many instances resistance genes against biotic factors are not even available in closely related species of cultivated fruit trees, but occur in wild species or non cultivated cultivars, which have only a poor fruit quality.

The application of molecular techniques makes new resistance genes available and it is imaginable that breeding steps will take less time, if desired traits can be directly introduced into high yielding cultivars, without transferring the entire genetic background, containing also undesirable traits.

The methods mainly applied for fruit tree transformation are (a) biological vectors, e.g. *Agrobacterium*-mediated transformation, (b) direct DNA transfer to protoplasts by chemical or electrical induction of plasmalemma permeability or (c) non-biological vector systems, especially micro-bombardment (Petri and Burgos 2005).

The use of *Agrobacterium* as biological vector benefits from the fact that fruit trees are within the host range of *Agrobacterium*. Furthermore this transformation method targets the T-DNA to the nucleus, leading to a stable integration into the host DNA, frequently in low copy numbers when compared to that obtained with biolistics (Mehlenbacher 1995; Potrykus, personal communication).

Particle bombardment has been applied efficiently for the transformation of papaya, peach and grapevine (Fitch et al. 1990; Yeh and Gonsalves 1994; Kikkert et al. 1996).

Fruit trees are considered to be recalcitrant material for molecular biology techniques, including genetic transformation (Gray and Meredith 1992; Pena and Seguin 2001). The regeneration of transformed plantlets has been recognized for many years as a major bottleneck in the transformation of fruit tree species (McGranahan et al. 1988). Although Haberlandt postulated the totipotency of plant cells, the high degree of differentiation of fruit tree tissues makes it more difficult to provide conditions permitting the explants to undergo dedifferentiation and to enter a new differentiation pathway (Laimer 2003b). Attempts to improve crop plants by genetic engineering techniques will therefore depend very strongly on the availability of reliable protocols for transformation, selection and regeneration. Furthermore regeneration of plants from single cells is a precondition for *Agrobacterium tumefaciens* mediated gene transfer to achieve homogeneously transformed plants (Polito et al. 1989).

Fruit trees are regenerated either via organo- or via embryogenesis. The choice of the best explant is a crucial decision, even today with many developed protocols: the genotypes interesting for transforming sometimes are poorly regenerating; transformed cells in an explant are not necessarily also the ones competent for regeneration.

Leaf discs and stem cuttings represent complex explants which allow regeneration of plantlets from many cultivars, also including woody species (Laimer et al. 1989).

Regeneration from petioli of *Vitis* rather seemed to give rise to chimeric regenerants, due to the fact that subepidermal and epidermal cells jointly contributed to an initiating promeristem (Colby et al. 1991).

Regeneration of fruit trees and grapevines was successful from embryogenic cultures (Perl et al. 1996; Kikkert et al. 1996; Gölles et al. 2000). Somatic embryogenesis definitively offers the advantage of single cell regeneration and therefore currently appears to be the most promising approach to introduce new genes in woody crop species (da Câmara Machado et al. 1995).

Reliable protocols for *Agrobacterium*-mediated transformation of stone fruit species also involve cotyledons of immature embryos at a certain stage of development after full bloom (Laimer da Câmara Machado et al. 1992) or hypocotyl slices as explant material (Mante et al. 1991).

The selection system for the recovery of transgenic fruit tree plantlets is a further crucial step. In poorly regenerating explants transformed cells may also die because of the isolation effect, if confronted with a high selection pressure from the beginning (Laimer 2003b). Among the most commonly used selection genes are neomycin phosphotransferase (*npt II*), conferring resistance to aminoglycoside antibiotics, and phosphinothrycin acetyl transferase (*pat*), conferring resistance to the herbicide phosphinotrycin (Miki and McHugh 2004). Kanamycin selection, widely used in screening for transformants, is known to have inhibitory effects on the regeneration capacity of somatic embryogenic cultures and leaf disc of grapevine, apricot, cherry, cocoa and other fruit trees (Colby and Meredith 1990; Laimer et al. 1992; da Câmara Machado et al. 1995; Gölles et al. 2000; Antúnez de Mayolo 2003).

Due to public acceptance concerns over antibiotic resistance marker genes (EFSA 2004), more recently positive selection strategies have emerged, like the use of transgenes able to utilize unusual carbon sources like xylose and mannose (Haldrup et al. 1998; Zhang et al. 2000), or encoding enzymes involved in hormone biosynthesis (Ebinuma et al. 1997, 2001; Kunkel et al. 1999). For papaya, Zhu et al. (2005) described a successful selection system using phospho-mannose-isomerase as selectable marker.

9.5.2 Description of Construct Design

Genetic engineering offers a means of incorporating new virus resistance traits into existing desirable cultivars. The expression of the viral coat protein

gene in transgenic plants induced similar protective effects as classical cross protection and was therefore distinguished as "coat protein-mediated" protection (Beachy et al. 1990). Since viral sequences encoding structural and non-structural proteins were shown to confer resistance, this concept was enlarged and termed pathogen-derived resistance (PDR) (Lomonossoff 1995).

Viral coat protein (CP) sequences have been used as sense, antisense, full length, truncated or untranslatable constructs. The level of protection conferred by CP genes in transgenic plants varies from immunity to delay and attenuation of symptoms, and in some cases the protection is broad and effective against several strains of the virus from which the CP gene was derived.

Pathogen-mediated resistance meanwhile has been shown to be RNA mediated and based on mechanism of co-suppression and post-transcriptional gene silencing (PTGS) or homology related gene silencing (Dougherty and Parks 1995; Wassenegger and Pélissier 1998). Sequence specific RNA silencing processes in plants point to the existence of a natural defence mechanism of adaptive protection against viruses (Voinnet 2001; Waterhouse et al. 2001; Baulcombe 2004). Furthermore, many plant viruses encode proteins suppressing PTGS, suggesting a co-evolution of defence and counter-defence between the host and the invading virus (Voinnet et al. 1999).

In the case of transgenic fruit trees, initially the use of translatable and non-translatable coat protein sequences yielded both immunity and recovery resistance in model plants; however both the number of protected lines as well as the level of protection against homologous virus strains seemed worthwhile improving. A further driving force for the modification of constructs were safety considerations concerning (a) selection of viral sequences reducing the potential risk of recombination or (b) mutations of the cp gene suppressing particle assembly, heterologous encapsidation and complementation (Balázs and Tepfer 1997; Varrelmann and Maiss 2000; Varrelmann et al. 2000).

Greene and Allison (1994) detected recombinant viruses only when the transgene had no deletions in the 3′NTR adjacent to the CP, concluding a possible reduced transgenic target length involved in prevention of recombination. Therefore it was suggested, that replication initiation sites should be excluded from transgene constructs (Allison et al. 1996). Mutation of the conserved amino acid motifs RG and D within the potyviral cp abolished the ability of particle formation (Varrelmann et al. 2000). To minimize even putative biological risks of virus-resistant transgenic plants, untranslatable viral genes would assure that no additional transgenic protein is produced in the plants, and in addition, the removal of specific functions of the viral protein by mutation can abolish the transfer of functions to challenging viruses via recombination (Varrelmann and Maiss 2000). This has been accomplished by a number of constructs containing a translatable or non-translatable cp-gene sequence of PPV NAT with and without the 3′NTR, that were first tested on

herbaceous hosts and finally transformed into *Prunus sp*. (Korte et al. 1995; Mendonça 2005).

The most recent development of constructs involves inverted repeats of viral coat protein- or replicase genes. A significant improvement of dsRNA mediated silencing compared to the originally used sense and antisense transgenes is that the dsRNA transgens produce large quantities of siRNAs and result in a much higher frequency of transformed plant lines that display efficient virus silencing (Smith et al. 2000). PTGS has been achieved with high efficiency in transgenic plants expressing self-complementary hairpin RNAs having two complementary regions that form a double stranded region separated by a short loop. Hairpin constructs having a spliceosomal intron inserted in the loop region show an efficiency of up to 90% in eliciting RNA silencing (Wesley et al. 2001). This approach has also been described in herbaceous plants for PPV (Pandolfi et al. 2003).

Truncated cp-sequences have been described to confer resistance, thus suggesting the use of partial cp-sequences for new constructs, possibly omitting the N-terminal region involved in aphid transmissibility and reducing the 3′NTR reducing potential recombination events. The use of these fragments as inverted repeat constructs with the ST-LS1 intron (Maiss, personal communication) is currently under investigation.

The constructs might be improved to avoid backbone sequences unexpectedly integrated, particularly in the case of pBin19 derived plasmids (Schiemann et al. 2003), by the use of other reduced and optimized vectors, e.g. pGreen (Hellens et al. 2000).

A further advantage is represented by temporally and spatially inducible gene expression by the use of tissue specific promoters. The pathogen inducible Mal d 1 promoter (Pühringer et al. 2000) might be an alternative to express PPV transgenes. In a first approach the inducibility of the *uidA* gene by PPV in *N. benthamiana* was analysed. Also herbaceous model plants carrying non-translatable versions of the cp gene of the non-aphid transmissible strain PPV-NAT under the control of the Mal d 1 promoter were regenerated (Mendonça 2005).

9.5.3 Survey of Virus Resistance in Transgenic Fruit Trees

Compared to various approaches experimented in herbaceous host plants, engineered protection in the case of fruit trees, there are only a few constructs and plant genotypes reported in the literature (Table 9.2), obviously due to the different time frame required for these experiments, but also due to the relative minor importance of fruit trees, when compared to staple crops or vegetables. In most of the cases indeed co gene sequences have been applied for construct design. Particular attention was given to potential ecological consequences in the case of GFLV by employing non-translatable and truncated cp-constructs (Gölles et al. 2000; Maghuly et al. 2005b). Considering the

Table 9.2. Survey of transgenic fruit trees transformed with viral cp (coat protein) genes. GCMV (grapevine chrome mosaic virus)

Virus	Host	Construct	References
CTV	*Citrus aurantifolia* Mexican lime	cp in sense orientation	Dominguez et al. (2000, 2002)
	Citrus aurantium	cp in sense orientation	Ghorbel et al. (2000)
	Citrus paradisi; Rio Grande	cp in sense orientation	Yang et al. (2000)
GFLV	Vitis vinifera; *Chardonnay*	cp in sense orientation	Mauro et al. (1995)
	Vitis vinifera; Russalka	cp in sense or antisense orientation, non-translatable cp, 5′TR cp and 3′TR cp	Gölles et al. (2000); Maghuly et al. (2005b)
	Vitis vinifera; Nebbiolo, Lumassina, Blaufränkisch	cp in sense or antisense orientation	Gribaudo et al. (2003); Gambino et al. (2005)
	Rootstock Richter 110 (*V. berlandieri* × *V. rupestris*)	cp in sense orientation	Krastanova et al. (1995); Laimer et al. (unpublished)
	Rootstock 41 B (*V. berlandieri* × *V. riparia*); *Rootstock SO4* (*V. vinifera* × *V. berlandieri*)	cp in sense orientation	Mauro et al. (1995)
	Rootstock RPG1	cp in sense or antisense orientation	Laimer et al. (unpublished)
ArMV	*Vitis vinifera* Russalka	cp in sense orientation	Gölles et al. (2000)
GVA	*Vitis vinifera* Russalka	cp in sense orientation	Gölles et al. (2000)
GVB	*Vitis vinifera* Russalka	cp in sense orientation	Gölles et al. (2000)
GCMV	*Rootstock Richter 110* (*V. berlandieri* × *V. rupestris*)	cp in sense orientation	Le Gall et al. (1994)
PPV	*Prunus armeniaca*	cp in sense orientation	Laimer da Câmara Machado et al. (1992)
	Prunus domestica Stanley	cp in sense orientation	Scorza et al. (1994)
PNRSV	*Prunus subhirtella*	cp in sense orientation	Laimer da Câmara; Machado et al. (unpublished)
PRSV	*Carica papaya* Sunset	cp in sense orientation	Fitch et al. (1992); Lius et al. (1997)

high economic impact of CSSV, it appears remarkable that so far there exists no report on transformation of cocoa against CSSV.

Although several transgenic fruit crops have been developed and evaluated in the greenhouse for virus resistance (Table 9.2), extensive field tests and commercial introduction have been lagging behind other traits and herbaceous host plants. Deregulation and subsequent commercialization have been achieved by APHIS/USDA in September 1996 only for the trans-

genic line 55-1 named 'SunUp', a 'Sunset' cultivar which is a sib of 'Sunrise', the most widely grown papaya cultivar worldwide (Strating 1996). Since the resistance of transgenic line 55-1 is not effective against PRSV isolates from other geographic origins (Tennant et al. 1994), other genes from PRSV isolates collected in the Caribbean, South America and Asia are being engineered and transferred into commercial papaya germplasms to develop plants resistant to PRSV strains outside of Hawaii (Gonsalves et al. 2004).

9.6 Conclusions

Transformation of fruit tree species can be accomplished and resistance demonstrated in a relatively short period; the agronomic evaluation of transformed plants for fruit production however requires many years. Therefore experiments need to be carefully planned. The genetic variability of individual viruses is an important consideration when choosing sources of genes, and when making evaluations of resistance, once transformation is achieved.

Although several efforts are underway to produce virus resistant fruit trees, papaya is the only one successful example to date that made its way into commercialisation. This is in part due to technical factors; however, there are also many legal and IPR issues behind this situation (Laimer 2005).

It should also be remembered that prevention and avoidance are basic components for the management of fruit tree viruses. Many of the current virus disease problems in fruit trees could have been prevented by eliminating the distribution and propagation of infected propagating materials, and by wiser use and movement of germplasm.

In the case of perennial trees challenging questions remain to be solved in future:

> What confers more sustainable protection: recovery, resistance or immunity?
> Should we go for prevention of local virus infection or systemic disease resistance?
> How can we achieve pyramiding of resistance genes in fruit trees?

And obviously the question on how to gain public acceptance for these products.

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10 The Use of Genetic Transformation Procedures to Study the Defence and Disease Resistance Traits of Trees

TREVOR M FENNING

10.1 Introduction

Trees are subjected to numerous physical injuries and assaults during their long lifetimes, some abiotic and some biotic. In order to thrive in face of these assorted stresses, trees need multiple and overlapping mechanisms to be able to detect and respond to them in an appropriate and timely manner. It is the intention of this chapter to review briefly what is known about these mechanisms in higher plants in general, how they are related to trees and finally how plant transformation techniques can be used to study them at the functional level.

Emphasis will be given to investigations of defense mechanisms that serve to protect trees against insect attack, and the role and biosynthesis of terpenoid biomolecules as an example of current work. However, some consideration will also be given to the results obtained when exotic defensive traits have been transformed into trees, such as the toxin genes from *Bacillus thuringiensis*, and to the use of modern genomic techniques to study the susceptibility and responses of trees to introduced pathogens, which are needed to guide future transformation studies.

10.2 Ecological Background

Trees are large perennial plants, which often live in dense stands that we call forests, and have the potential to accumulate large populations of pests and diseases. Ecological mechanisms normally prevent this, but even occasional mass pest or disease outbreaks can threaten entire forests. The environmental and economic damage that such outbreaks can cause is not unlike that of a forest fire, in that many years of accumulated growth can be lost in a short time (Raffa 1989).

The sheer longevity and size of trees are the critical determinants of their ecological survival strategies in response to pests and diseases. The

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importance of this issue cannot be over-emphasized, because most studies into plant survival and defense traits have been performed with short lived model plants and/or crop species, which are of uncertain relevance when studying the defense and ecological responses of trees. The success of efforts to breed faster growing or better trees may be severely undermined if they are inadvertently rendered more susceptible to biotic stresses, so this matter needs to be given serious consideration at the beginning of any tree improvement program and not treated as an afterthought.

Crop plants have been subjected to intensive breeding by humans for millennia for our own objectives, and farmers are principally concerned about the overall ability of their crops to compete at the field level rather than for individual plants. It is therefore to be expected that the defense and stress responses of crop plants will have been heavily disrupted as compared to wild plants, just as the scents of ornamental flowers are often impoverished compared to their wild relatives (Pichersky and Gershenzon 2002; E. Pichersky, personal communication).

In addition, pests and stresses that afflict crop plants, which naturally attract the attention of agriculturalists, often owe more to the details of agricultural practice and the inadvertent transfer of herbivores pests during human migrations than to what the ancestors of these plants may have had to face in wild situations. As a result, considerable caution is needed when comparing the extensive work on the diseases and stress responses of crop plants, to the results that may be obtained with wild plants of any kind.

However, caution is still needed when comparing results with wild annual or herbaceous plant systems to trees, due to the literally very large differences in their life habits. It is most likely that all the basic primary physiological and biochemical mechanisms of trees will be homologous to those of easier to study model plants such as arabidopsis (Kirst et al. 2003; Groover 2005), but the details of their secondary metabolic processes and ecological survival strategies are likely to be as divergent from those of smaller plants as any other aspect of their appearance.

In particular, because trees are large and long lived, it is clear that they cannot hope to evade herbivores or diseases, because when such organisms locate a suitable host tree, the only requirement for them to find abundant food for many generations thereafter is to stay there. This is quite unlike the situation facing the natural herbivores of annual plants, which have to contend with an intermittent food supply that re-appears somewhere else every year. It might be expected, therefore, that trees would accumulate more and more herbivores and pathogens over time, and dense stands of trees of the same species especially so, to the point where one might logically expect their survival to be at risk.

A reliance on direct defenses alone is therefore unlikely to be sufficient to ensure a trees' survival in the face of such biotic threats, as the much more rapid generation times of the pest species would enable them to quickly overcome such brute force defensive strategies, immediately followed by a

mass infestation once this occurred (Raffa 1989, 2004). That this does not usually happen indicates that other defensive mechanisms may be highly developed in tree species, possibly involving a combination of sophisticated defense mechanisms and ecological partnerships that serve to keep tissue losses due to herbivory down to tolerable levels (Whitham et al. 1991; Haukioja 2005).

Identifying what these 'tree specific' defense or ecological mechanisms are, and how they function at the biochemical and genetic levels, is a formidable challenge that is only just beginning to be addressed. It is the aim of this chapter to review briefly the uses and limitations of plant transformation for studying these issues, as well as how this effort is being complimented by other newly developed technologies.

10.3 The Constitutive and Induced Defenses of Plants

It is not possible here to give this rapidly developing subject more than a cursory overview, but some appreciation of the defense and signaling mechanisms of plants is needed, both to understand the particular challenges facing tree biologists working in this field, as well as the exciting scientific opportunities that studying the molecular-genetic defense mechanisms of trees affords.

Plant defenses are usually divided into the constitutive and the induced, with constitutive defenses representing anatomical or biochemical features that are preformed without any prior exposure to a pest or pathogen (Wittstock and Gershenzon 2002, Sect. 10.3.1), while induced defenses are biochemical or physical changes in a plants phenotype that are activated in response to some form of stress or biotic attack (Sects. 10.3.2 and 10.3.3). A recurring theme here is that the diversion of resources towards defense can be costly in terms of reproductive success; therefore it is advantageous to invest only minimal resources in defense so long as herbivores or pathogens are not causing problems (Karban and Baldwin 1997; Baldwin 1998; Heil 2002), albeit with the risk that in the event of a sudden attack serious damage may be incurred before an appropriate defense is activated.

10.3.1 Constitutive Defenses

Although, the induced defense responses of plants are currently attracting most interest, constitutive defenses are probably at least as important to a plants survival and are often synergistic with their induced responses. However, because these structures are always present, it is scientifically problematic to demonstrate unequivocally that they are truly defensive in nature, rather than serving some other purpose within the plant. Indeed, some

constitutive defenses in plants may well serve multiple purposes, for instance the thick bark present on mature trees protects the sensitive living tissues underneath from biotic and abiotic sources of damage, such as fire (Hudgins et al. 2003a; Franceschi et al. 2005). Examples of other constitutive defenses often found in trees may include fibers, thorns, chemical toxins and repellents, resins and thickened cell walls, although some of these features are also inducible. Even lignin can be considered as a defensive trait, as it is very tough and very few organisms can break it down, so limiting the number of organisms that can threaten a trees vascular system or trunk.

The use of transformation approaches to modify these features one step at a time has the potential to demonstrate their role within and to the plant, but needs to be used in conjunction with other approaches, as the defensive structures and responses of plants are likely to be complicated and overlapping and also very variable in wild plants, making a dissection of their function far from easy.

Two other issues also need to be kept in mind when studying plant 'defenses', the first being merely because a structure appears to be unambiguously defensive in nature, e.g. thorns, or is clearly induced in response to herbivory does not mean that it is always effective, especially against specialist or introduced pests or pathogens. Second, when a plant is attacked by herbivores and/or pathogens, it not only needs to defend itself, but it also needs to adjust its metabolism and physiology to accommodate the disruptions they are causing to its normal growth processes. Because of this, there are probably a lot of 'house-keeping' actions that go on inside a plant when something is eating it that are only indirectly related to any attempt to defend itself, such as temporarily shutting down photosynthesis perhaps (Sect. 10.4). Presumably, however, plants need to be able to do both successfully to continue to survive the numerous biotic challenges they surely face every day in the natural environment.

One additional point about constitutive defenses that needs to be mentioned is that they are usually thought of as only functioning 'directly' against herbivores or pathogens by exerting a direct physical or chemical effect on the individual organisms that come into contact with them. An example of a constitutively expressed indirect defense has recently been found in several *Acacia* species from central America, which continually secrete extra floral nectar which attracts aggressive ants to live on them (Heil et al. 2004).

The most obvious constitutive defense of trees, however, is their bark. The roles of bark and terpenoid resin defenses of conifers against insect attack have been reviewed by Trapp and Croteau (2001) and Franceschi et al. (2005), and the readers of this chapter are directed to give these articles their full attention, but some further discussion of these issues is given in Sects. 10.6 and 10.8.2. Amongst much else, Franceschi et al. (2005) describes the numerous physical structures in conifer bark that present a multifaceted and synergistic array of mechanical and chemical defenses that are effective in

deterring all but the most specialized insect herbivores, which is presumably also the role of bark in non-coniferous trees. These complex structures are likely to defy any 'simple' gene by gene reductionist analysis aimed at determining their precise defensive function for some time to come.

10.3.2 Induced Direct Defenses

The induced defenses of plants are those that increase in efficiency or abundance in response to an injury or after exposure to specific herbivore elicitors, and are usually sub-divided into the direct and the indirect (Sabelis et al. 1999). They serve to protect the plant from herbivores either 'directly' by the production of as diverse an array of toxins and feeding inhibitors as there are plant species (Duffey and Stout 1996; Baldwin and Preston 1999; Baldwin et al. 2001) or 'indirectly' by attracting predators or parasitoids from the third trophic level (Sect. 10.3.3).

There is not the space in this chapter to discuss more than superficially the extensive literature relating to the induced direct defenses of plants, and what we know about how they relate to trees, but the readers of this chapter are directed to the book by Karban and Baldwin (1997) for an in-depth discussion of induced plant defenses in general and the chemical and ecological principles associated with them, to Franceschi et al. (2005) for a recent review of the resin based defenses of conifers and to Haukioja (2005) for a discussion of the defensive responses of angiosperm trees to attack by leaf beetles.

The resin systems of conifers have long been thought to be of defensive value (Berryman 1972), and in the Pinaceae at least are believed to function either by gluing up or physically expelling insects that rupture a charged resin canal (Trapp and Croteau 2001), as well as being distasteful to non-specialist herbivores of all kinds. It has been noted that even specialist stem boring insect herbivores of conifers either seek to avoid puncturing resin canals, at least in the early phases of an attack, e.g. the white pine weevil (Boucher et al. 2001), or normally avoid healthy trees which presumably have fully charged resin canals e.g. bark beetles (Berryman 1972; Franceschi et al. 2005).

However, it has also been observed that the conifer species with the most elaborately developed resin canal systems also attract the attention of the most lethal bark beetle species (Hudgins et al. 2004; Franceschi et al. 2005), perhaps underlining the point that such direct defensive strategies on their own are of potential traps for perennial organisms over evolutionary time scales, as specialist herbivores will always find ways to bypass them. Despite this possible handicap, however, the Pinaceae includes among its members some of the most abundant and widely distributed tree species in the world.

Indeed, Haukioja (2005) notes that after many years studying the mountain birch (*Betula pubescens*) in Scandinavia and its many putative 'directly' defensive compounds, including many types of phenolics, it appears that they have only a very slight influence on the population fluxes of its specialist

defoliating beetle *Epirrita autumnata*. Accumulating toxic secondary metabolites may be a good survival strategy for short lived annuals, or for deterring generalist herbivores from eating more tissue than a tree can afford to lose, but this evidence appears to emphasize the notion that this is not a viable strategy for long lived perennials to deter specialist herbivores, as they will always find ways to circumvent such compounds and even utilize them to enhance their own survival, and become a greater threat than would otherwise be the case.

Instead, the eruptive outbreaks of *E. autumnata* on stands of *B. pubescens* seem to be the result of a subtle ecological interplay between this specialist herbivore and its predators and parasitoids from the third trophic level, which are in turn mostly influenced by the trees 'indirect' defensive responses (see Sect. 10.3.3). Haukioja (2005) also emphasizes that short and long term changes to the nutritional status of many trees occur in response to intensive herbivory. A similar response was recently observed for *Pinus sylvestris*, which reduced its rate of photosynthesis and its RUBISCO protein levels in response to egg deposition by an insect herbivore (Schröder et al. 2005). What the precise value to a tree for such behavior is as yet unclear, but presumably it does have some defensive function, as may other so called 'house keeping' functions, as discussed in Sects. 10.3.1 and 10.4.

The last point that will be made here about the induced defensive responses of trees is that, although it makes a compelling case for the defensive role of selected pathways if they specifically activate in response to herbivory, such evidence remains only correlative if one does not know the mode of action of the molecules concerned. For instance, it appears that at least some proteases and protease inhibitors serve to modulate a plants receptor and systemic signaling after wounding or attack by pathogens, rather than acting directly as defensive compounds (Dodds and Schwechheimer 2002; Takayama and Sakagami 2002; Sect. 10.4).

In comparison, because of the commercial and medical interest in nicotine, its precise biosynthetic and induction pathways in *Nicotiana* ssp. are well understood, as is its exact mode of toxicity on animals of all kinds, which is why it is a favorite for ecological studies (Baldwin and Preston 1999; Baldwin et al. 2001). The same level of certainty cannot be applied to most putative defensive compounds produced by trees, especially if reliably analyzing them is problematic as with phenolics, or if they appear to have little if any effect on the herbivores of interest (Haukioja 2005). Transformation approaches are therefore invaluable for studying the role of the genes associated with the defensive pathways of trees.

10.3.3 Induced Indirect Defenses

The induced indirect defences to arthropod herbivores function by attracting predators or parasitoids from the third trophic level, via the emission of

specific volatile, mostly terpenoid signals that are released in response to insect feeding or egg deposition. The release of these volatile blends has been interpreted as a "cry for help" by the plant to predators or parasitoids, which then kill the herbivores and thus reduce the damage done (Sabelis et al. 1999; Degenhardt et al. 2003; Hilker and Meiners 2006), and may enhance the effectiveness of other toxins (Guillet et al. 1998; Pichersky and Gershenzon 2002).

Although this phenomenon was only discovered relatively recently (Turlings et al. 1990, 1995), perhaps it should not have been a surprise as many flowering plants use similar volatile mixes to help attract insect pollinators (Pichersky and Gershenzon 2002), suggesting an ancient origin for volatile signalling in plant-insect relationships (Gershenzon and Kreis 1999).

The literature relating to the induction of plant volatile release in response to insect feeding is rapidly expanding, with responses from numerous species having been reported, including cotton, lima bean, tomato, maize, arabidopsis as well as some wild and cultivated *Nicotiana* species (Sabelis et al. 1999; Baldwin et al. 2002; Kessler and Baldwin 2002; Degenhardt et al. 2003; Dudareva et al. 2004). It was even proposed as long ago as 1983 from work with trees (Baldwin and Schultz 1983) that insect damage specific signals from one plant might be capable of alerting neighboring plants to an impending attack.

Although the idea remains controversial in relation to annual plants (Pickett and Poppy 2001; Dicke et al. 2003), as it has been argued that there is no advantage to the emitting plant in alerting a neighboring competitor to the fact that it is under attack (Baldwin et al. 2002), but more persuasive evidence came from ecological studies with forest trees, possibly because perennial plants are certain to end up accumulating the same insect pests as their neighbors (Haukioja 2005).

Volatile emissions from wounded plants, and plant scents in general, commonly include many mono- and sesqui-terpenes, but are not limited to them (Dudareva et al. 2004; Sect. 10.8.1), the biosynthesis and genes for which are highly conserved (Sect. 10.8.2; Gershenzon and Kreis 1999; Pichersky and Gershenzon 2002; Martin et al. 2004). It should be noted, however, that plants also increase their emission of volatiles in response to various abiotic stresses such as excess heat and drought, as well as in response to insect herbivory and pathogens (Logan et al. 2000). This suggests that the emission of these compounds may have originally evolved for some reason associated with a plants primary metabolism (Dudareva et al. 2004), which insects subsequently learned to cue on early in their evolution, and so leading to the highly intertwined relationship that we see today between insects, plants and their volatile emissions.

The ecological and metabolic costs and benefits of these induced indirect defenses have been frequently addressed (e.g. Karban and Baldwin 1997; Baldwin 1998; Sabelis et al. 1999; Heil 2002), but it seems probable that inducing extra defenses according to need is 'cheaper' for the plant than constitutively expressing them and that induced indirect defenses are even

more cost effective, as analyzed metabolically by Gershenzon (1994). Not insignificantly for long-lived trees, a combination of induced direct and indirect defenses are probably much more difficult for insect herbivores to overcome than constitutively expressed direct defenses alone are (Franceschi et al. 2005; Haukioja 2005).

It should not always be assumed that volatile emission by plants in response to insect damage is advantageous, as there are several examples known from forest ecosystems of extremely damaging 'eruptive' insect herbivores that use these emissions as aggregation cues to initiate mass attacks (Haukioja 2005). Many bark beetles modify terpenoids consumed from their host to do exactly this (Franceschi et al. 2005; Sect. 10.6), and males of the forest cockchafer beetle *Melolontha hippocastani*, cue on the emissions of trees infested with females in order to find their mates (Ruther and Hilker 2003).

A further complication to the theory of plants 'calling for help' from the third trophic level is that predators and hyper-parasitoids from the fourth trophic level also presumably cue on the same emissions, reducing their defensive value (Arimura et al. 2005). Furthermore, some parasitoids might cause their hosts to consume more plant material rather than less.

The overall cost and benefit of these defense strategies is therefore not always clear cut nor one way, but it seems likely that trees are playing an even more subtle version of "ecological poker" than has been observed with annual plants (Harrison and Baldwin 2004), and while they may not win every hand, they win sufficiently often for the tree habit to be successful (Haukioja 2005). The point has been well made by Ken Raffa (1989, 2004) that attempts to tip such complex ecological balances in directions that we may prefer for the tree species that interest us, by genetic engineering trees for pest resistance for instance, could result in negative consequences unless the relevant ecological parameters are evaluated very carefully first.

10.4 Wound Perception and Signaling

Studies into the mechanisms of volatile emission in plants revealed that herbivory induces them to be released locally at the site of damage and then systemically from undamaged leaves adjacent to those that have been eaten. Soluble compounds transported in the phloem or xylem, gases dispersed by diffusion in the apoplast, as well as electrical and hydraulic signals transmitted by the vascular system have all been suggested as mediating systemic induced resistance against insect attack (Malone 1996; de Bruxelles and Roberts 2001). However, it is still not entirely clear which are the genuine signals responsible for plants systemic responses, nor how they regulate volatile emission (Orians 2005).

The "myriad plant responses to herbivores" have been excellently reviewed by Walling (2000), and several studies have addressed the question of how

plant responses to herbivory are controlled at the molecular level (Arimura et al. 2000; Baldwin et al. 2001; Kessler and Baldwin 2002; Fäldt et al. 2003a; Belkhadir et al. 2004). In particular, the salicylic acid-dependent and jasmonic acid/ethylene-dependent signalling pathways have been given much attention (Kunkel and Brooks 2002).

It has been repeatedly demonstrated that the exogenous application of jasmonic acid (JA) to plants causes them to emit volatile blends similar to those released in response to insect feeding, e.g. on conifers (Franceschi et al. 2002; Hudgins et al. 2004), and also for elms (Sect. 10.5). Jasmonic acid is produced via the octadecanoid pathway from linolenic acid, and its role as wound signal has been demonstrated in many plant systems (Karban and Baldwin 1997; Farmer et al. 2003), although it should be noted that jasmonates have many other roles in plants other than just wound signaling (Creelman and Mullet 1997; Cheong and Choi 2003).

In addition, many compounds other than jasmonic acid are involved in mediating plant defense responses (Arimura et al. 2005), including: changes in free calcium and oxidative bursts at the site of injury (Nürnberger and Scheel 2001; Moran et al. 2002); various other forms of jasmonates, including the volatile methyl jasmonate (Cheong and Choi 2003; Farmer et al. 2003); lipid based compounds other than jasmonate (Weber 2002; Farmer et al. 2003); ethylene and salicylic acid (Pieterse and van Loon 1999; Wang et al. 2002); systemin and prosystemin or possibly other oligo-peptides (Ryan et al. 2002; Takayama and Sakagami 2002); and oligosaccharides (Nürnberger and Scheel 2001).

From all this, it should be clear that a plants complete response to insect attack is very complex indeed and that jasmonates are probably not involved in the initial detection and local response to insect attack, but rather are components of the long-distance signals emanating from the region of the attack, stimulating appropriate systemic responses in the rest of the plant (Stratmann 2003). Farmer et al. (2003) made the interesting suggestion that plants probably use different signal compounds or signal ratios rather like musical notes or codes, each specific to particular types of injury. Insect herbivores also attempt to evade or manipulate such signals, presumably to confuse their host plants defense responses (Dodds and Schwechheimer 2002; Arimura et al. 2005), which might explain the finding that the eggs and neonate larvae of many lepidopteran insects contain significant concentrations of jasmonic acid (Tooker and de Moraes 2005).

Although there is now a substantial literature relating to plant wound signaling as briefly summarized above, it needs to be emphasized that it is still far from clear how such signals are physically transmitted around a plant in anything resembling a coherent manner (Malone 1996; Orians 2005), let alone in anything as large as a tree. Furthermore, it should be noted that these studies have been conducted using many different plant species, often with crop plants and their pests, which may be responsible for some of the apparent contradictions in the literature relating to plant defense responses

and signaling. This underlines the importance of comparing results within each species, and then contrasting the data to results obtained with wild species such as trees and their natural herbivores, before trying to draw wider conclusions.

The defensive responses of field elms to egg laying by the specialized leaf beetle *Xanthogaleruca luteola* is an especially instructive example. The localized scratching on leaves by gravid female beetles prior to egg laying does not elicit the emission of a specific volatile blend from the tree, nor does similar scratching with a scalpel blade, but the application of egg masses to these scratches does. Furthermore, since watering cut elm twigs with jasmonic acid stimulated the emission of a similar volatile blend to that which occurs in response to egg laying by *X. luteola*; this implies that scratching alone does not result in a sufficient release of jasmonate to initiate a defense signal within the plant, but that a specific elicitor is additionally required.

From this, it is reasonable to propose that there must be a network of receptors and molecular regulators initiating and controlling systemic wound signaling within plants (Wasternack and Parthier 1997; Moran et al. 2002; Morris and Walker 2003; Rakwal and Agrawal 2003; Arimura et al. 2005; Lorenzo and Solano 2005). Indeed, the sensitivity of plants to potential insect herbivores was further demonstrated by Bown et al. (2002), who showed that within seconds of herbivorous insect larvae merely walking over tobacco and soybean leaves, changes in cellular Ca^{2+} levels occurred, along with an oxidative burst and an increase in chlorophyll fluorescence, finally resulting in local increases in putative defense signaling compounds.

This is particularly interesting because similar changes in Ca^{2+} levels along with an electrical depolarization were observed by Maffei et al. (2004), in lima bean leaves that had been partially eaten by the Mediterranean cutworm *Spodoptera littoralis*. These effects spread through the attacked leaf and preceded the plants' systemic responses.

Such changes in a chewed on leaf's electrical potential could be the first local or systemic signal of insect related damage as suggested by Maffei et al. (2004) but contradicting previous data (Malone 1996), or be occurring as a consequence of an abrupt de-coupling of the photosynthetic mechanisms, which would also explain the apparent increase in leaf fluorescence observed by Bown et al. (2002).

It is not obvious why photosynthesis should be disengaged quite so promptly in response to herbivore contact, unless the maintenance of the redox potentials it generates interferes with wound signaling, if changes in the electrical potentials of wounded leaves really are signals (contrary to Malone 1996). Alternatively, some secondary metabolic compounds are induced when plants are exposed to insects are produced in the plastid, possibly necessitating a diversion of the organelles biosynthetic machinery to such purposes.

Despite these uncertainties, however, it is still viable to hypothesize that networks of surveillance receptors are activated in plants by wounding and

exposure to elicitors characteristic of the insects or pathogens (Morris and Walker 2003; Parker 2003; Arimura et al. 2005), presumably in the first instance immediately proximal to the site of any damage. Depending upon the precise permutation of receptors activated, these then initiate a cascade of local tissue responses, including fast defense responses and the release or amplification of a systemic signal code by neighboring tissues, which then activate systemic acquired resistance mechanisms in the rest of the plant (Arimura et al. 2005), as appropriate and as the plant is capable of producing. Presumably there must also be some means of measuring the signals initiated, "cross-talk" between the different signal pathways in the event of complex situations (Maleck and Dietrich 1999; Kunkel and Brooks 2002; Lorenzo and Solano 2005), and some kind of feed-back mechanism to attune the defensive response according to need and damp out old signals (Arimura et al. 2005).

This said, however, Mithöfer et al. (2005) observed that patterns of volatile release in lima bean plants occurring in response to repeated small scale mechanical damage that mimicked herbivore feeding patterns were very similar to those released after actual herbivory by *S. littoralis* or the snail *Cepaea hortensis* or methyl jasmonate treatment. This may indicate certain generalized defense responses can still be activated even in the absence of specific herbivore related signals, provided they are sufficiently activated and/or if repeated small doses of jasmonates accumulate as can be released by mechanical wounding (Arimura et al. 2005). Herbivores and pathogens also come in many combinations other than just insects alone (Karban and Baldwin 1997; Maleck and Dietrich 1999), and so a plant must be capable of responding in a coordinated manner to all the biotic and abiotic stresses that it is likely to encounter during its lifetime.

Nevertheless, a sophisticated network of receptors is clearly important for activating and controlling plant defense and signaling responses (Kunkel and Brooks 2002; Rathjen and Moffett 2003; Morris and Walker 2003; Lorenzo and Solano 2005). The most studied examples are the 'gene for gene' resistance/avirulence responses of *Pseudomonas syringae* when it attacks arabidopsis or tomato (McDowell and Woffenden 2003; Parker 2003).

The 'avirulence' determinants of *P. syringae*, often proteases, are probably better termed 'elicitors', which if detected by the host plant through its network of receptors can initiate a defense response, although similar phenomena are being studied in other plant pathogen relationships too, including for viruses and fungi (Parker 2003). If the host lacks the appropriate receptor and/or the pathogens suite of elicitors has changed, then it will not be detected and the attack will probably succeed (McDowell and Woffenden 2003).

Five classes of these putative receptors are recognized, in addition to protein kinases which are thought to be involved in regulating their activity (Rakwal and Agrawal 2003; Morris and Walker 2003). Literally hundreds of these putative 'R' genes and related receptors have been identified in arabidopsis, only a

handful of which have been assigned even a putative function (Nürnberger and Scheel 2001; Belkhadir et al. 2004). Long lived organisms such as trees with their ecologically complicated lifestyles, are hardly likely to have less.

Many of these are probably necessary for plants to regulate their own wound and other systemic signals, but others are probably specific for the more common microbial pathogens and insect herbivores (Morris and Walker 2003; Parker 2003). The precise suite activated is presumably responsible for each observed response, including systemic acquired resistance and the release of herbivore specific volatile blends (Arimura et al. 2005). This theory partially explains why in the event of being exposed to a novel insect herbivore or pathogen, trees often respond in an ineffectual manner or even not at all (Sect. 10.10; Dodds and Schwechheimer 2002; McDowell and Woffenden 2003).

For all these reasons, the genes responsible for these receptors and related signaling pathways, are likely to be of great interest to those working on the defense responses of plants and trees for some time to come. However, because they are likely to be constitutively expressed, associating them definitively with a particular foreign organism will be as difficult as for any other constitutively expressed defense trait. Indeed, since receptors are the very modulators of the defense responses and signaling cascades, their interactions are likely to occur at the protein-protein and protein-ligand level, which are much harder to study than changes in gene expression, therefore unraveling the full extent of their role will be problematic for some time to come, even for arabidopsis.

10.5 The Elm Leaf Beetle System

The elm leaf beetle system offers a rare and fascinating insight into how sophisticated trees' indirect defense responses can be, as well as indicating the likely future direction of research efforts to understand them at the molecular-genetic levels.

Within its native range, the European field elm (*Ulmus campestris*) is regularly attacked by the specialized monophagous Chrysomelid elm leaf beetle *Xanthogaleruca luteola*, which can occasionally defoliate whole trees (Kwong and Field 1994). Meiners and Hilker (1997, 2000) conclusively demonstrated that egg laying by *X. luteola* onto elm leaves was sufficient to induce defensive responses in *U. campestris*, including the systemic release of a distinctive blend of volatiles, attractive to the eulophid wasp *Oomyzus gallerucae*, which exclusively parasitizes the eggs of these elm leaf beetles, killing them in the process. These volatile emissions continue for about five days (T. Meiners, personal communication), closely matching the incubation time of the *X. luteola* eggs.

It had been hypothesized previously that plants might be able to distinguish between different life stages of their major natural insect herbivores, so as to induce the most appropriate defensive responses (see Sect. 10.3.3).

However, this was the first clear example of a stage-specific blend conferring a clear ecological advantage, free of the complications of interpretation that afflict work with agricultural crops and their pests. In addition, it was the first demonstrated example of a plant inducing an indirect defence to the presence of insect eggs alone (Hilker et al. 2002b).

Although a few other plant-insect systems have since shown similar behaviour, one of these is also with a tree, where egg deposition on *Pinus sylvestris* needles by the pine sawfly *Diprion pini* causes an increase in the emission of volatiles attractive to the egg parasitoid *Chrysonotomyia ruforum* (Hilker et al. 2002a), underlining the importance of such indirect defences to trees (Haukioja 2005).

The exquisitely attuned nature of the responses of *U. campestris* to egg laying by *X. luteola* has become even more apparent as more information is obtained about it: In order to attach each batch of 20–30 eggs to the surface of a suitable elm leaf, gravid leaf beetle females make a shallow groove on the epidermis with their mouthparts, and then glue their eggs onto it with the aid of a special oviduct secretion. It appears that some component of this secretion is responsible for activating the elm plants egg-specific responses, as scratching with a scalpel or by the insect does not elicit any response, but rubbing the isolated secretion onto a freshly made scratch does (Meiners and Hilker 2000), suggesting a highly evolved elicitor-receptor relationship (see Sect. 10.4).

Furthermore, other studies have revealed that the oviduct secretion of *X. luteola* does not elicit any similar response in the mountain elm (*U. glabra*), which is not the normal host plant for this insect and neither does the oviduct secretions of other closely related, but non-field elm eating Chrysomelid leaf beetles render *U. campestris* attractive to *O. gallerucae* (Meiners and Hilker 2000), underlining the extraordinarily specific and ecologically adapted nature of the *U. campestris-X. luteola-O. gallerucae* system.

In order to study this system in the necessary detail, the Max Planck Institute for Chemical Ecology, the Free University in Berlin and the University of Arizona are currently pursuing a collaborative project to collect RNA samples from relevant field elm tissues after egg laying and feeding treatments with *X. luteola* for more detailed analysis. Selected genes found to be associated with the ecological responses described here, will be transformed back into *U. campestris* for over-expression as well as RNAi repression, so as to probe their ecological function.

10.6 Bark Beetles and the Resin Defenses of Conifers

The most serious 'eruptive herbivores' are bark beetles (*Scolytidae*), which are probably the most important natural killers of trees worldwide, including conifers (Berryman 1972). Bark beetles can be found even in healthy forest, but normally only at low abundance. The details vary between species, but

adult beetles usually target old and dying trees, boring into the bark to lay their eggs, where the larvae then hatch and eat into the cambial and phloem layers before emerging as adults. Many bark beetle species also have highly specialized symbiotic associations with pathogenic fungi, which are carried into the tree by the beetles, where they attack the trees vascular tissue, weakening it further (Berryman 1972; Seybold et al. 2000; Franceschi et al. 2005).

Occasionally, however, bark beetles may be present in larger numbers, and individual beetles then use aggregation pheromones to initiate mass attacks on otherwise healthy trees to overcome their defenses (Wallin and Raffa 2004). In ideal conditions, there can be several generations of bark beetles a year, leading to a local population explosion of beetles, which can then spread out to devastate larger and larger areas of otherwise healthy forest, before the outbreak becomes self limiting due to insufficient trees remaining to support further population growth. A few apparently resistant trees may survive even in the worst affected areas, however.

One of the most widespread conifer species in northern and central Europe is *P. abies*, and its most damaging trunk boring bark beetle is *Ips typographus* along with its principle symbiotic pathogen, the spruce 'blue-stain' fungus *Ceratocystis polonica* (Berryman 1972). Conifers are evolutionarily distinct and predate woody angiosperms by a considerable margin (Kirst et al. 2003; Martin et al. 2004), so it is likely that bark beetles arose on them first and only spread onto angiosperm tree species much later. Thus, it is likely that the relationship between *P. abies*, *I. typographus* and *C. polonica* is very ancient and highly co-evolved, and so these trees may have defensive adaptations not found in the better characterized angiosperms (Walling 2000).

Perhaps the best known example of a putative defensive mechanism in conifers is their production of oleoresin, which consists primarily of terpenoids and accumulates either in specialized resin ducts or blisters (Berryman 1972; Hudgins et al. 2003b; Franceschi et al. 2005). The oleoresin of conifers consists almost entirely of terpenes and makes a very sticky glue like substance that sets hard once the volatile components have evaporated away (Trapp and Croteau 2001; Sect. 10.8.2).

A more recent discovery, however, is the existence of so-called 'phloem parenchyma cells' (or PP cells), which appear to contain concentrated phenolic compounds and form a more or less contiguous ring within the inner bark or outer phloem layers (Franceschi et al. 1998, 2000), and have been found in all conifer species investigated (Hudgins et al. 2003b). Any pathogen or insect attempting to penetrate the trees bark must pass through one or more of these cell layers and, in the Pinaceae at least, are also likely to rupture their network of resin canals, resulting in a sudden release of resin into the wound site, capable of evicting the pathogens or insects entirely, or entombing them in situ (Franceschi et al. 2005).

Various theories have been put forward to explain the variations in the susceptibility of individual trees to attack by bark beetles, including differences in terpene or phenolic content (Lieutier et al. 2003; Franceschi et al. 2005),

differences in their bark (Hudgins et al. 2003a), or the different growing conditions of individual trees (Sandnes and Solheim 2002). The reason(s) why some trees survive and neighboring ones do not is still unresolved, however (Wallin and Raffa 2004; Franceschi et al. 2005).

What is clear, however, is that *P. abies* has a large and powerful induced response to attacks by *I. typographus* and *C. polonica* and that this can render those trees that survive more resistant to subsequent attacks (Christiansen and Krokene 1999). For instance, after being wounded, members of the Pinaceae divert the development of some of their immature xylem vessels into forming so called traumatic resin ducts, in addition to their pre-existing constitutive resin duct system (Nagy et al. 2000; Krokene et al. 2003), as well as forming extra layers of PP cells (Franceschi et al. 1998, 2000). In short, these responses represent a novel form of acquired resistance, and consequently may have novel genes associated with them.

There will probably also be similarities to the defense and signaling responses of other plants, however, as it was shown recently that similar phenotypic changes can be stimulated by spraying spruce trees with methyl jasmonate (MJ), although its physiological activity in conifers is unknown (Kozlowski et al. 1999; Franceschi et al. 2002; Martin et al. 2002; Hudgins and Franceschi 2004). Furthermore, Zeneli et al. (2006) succeeded in demonstrating that a pre-treatment with MJ enhanced the resistance of *P. abies* to subsequent inoculations with *C. polonica*, although this was not tested in combination with bark beetles.

The Max Planck Institute for Chemical Ecology, the Canadian Forest Service, the Norwegian Forest Institute and the University of British Columbia are pursuing a joint program of field work with the *I. typographus* and *C. polonica* disease complex, including gene expression and biochemical profiling of resistant and susceptible clones, with and without pre-treatment by elicitors, including the use of laser dissection microscopy for isolating specific cell types for detailed analysis. Genes will be selected for further study by subjecting cDNA samples derived from plants exposed to *I. typographus* and/or *C. polonica* to micro array analysis, a subset of which will be transformed back into *P. abies* following the strategy discussed in Sects. 10.8.2 and 10.8.3.

It is to be hoped that, by applying this approach to the study of the signaling pathways and genes associated with exposure to *I. typographus* and *C. polonica* in the different lines of *P. abies*, it will be possible to determine the role and ecological functions of its defensive reactions and in so doing gain an important insight into why some trees are susceptible to attack by *I. typographus*, *C. polonica* while others are apparently resistant.

10.7 Other Pest Syndromes of Conifers

Bark beetles are not the only insect herbivores that afflict conifers which may be influenced by their resin defenses. Another example is the white pine

weevil (*Pissodes strobi*), which is a generalist herbivore of *Pinus* and *Picea* species across much of North America, and is particularly prevalent when many immature saplings are growing close together as can occur in plantations or regenerating areas of natural forest recovering from clear felling or fires. Female weevils lay their eggs under the bark of growing apical shoots of juvenile trees, and the larvae then proceed to eat the cambium, girdling and killing it (Boucher et al. 2001; Miller et al. 2005).

As with the susceptibility of *P. abies* to bark beetle attack, there are variations in the susceptibility of individual trees to attack by *P. strobi* and weevil performance, which in this case seem to be influenced by the initial density of resin canals in the host trees (Alfaro 1995; Boucher et al. 2001), suggesting that the resin canal systems of these trees might be capable of conferring at least some resistance. Several studies subsequently demonstrated that changes in the resin composition occurs in the terminal leaders of spruce trees in response to mechanical damage, MJ treatment and even to weevils themselves (Tomlin et al. 2000; Miller et al. 2005), encouraging the belief that perhaps by manipulation or pre-inducing these pathways it might be possible to improve the resistance of trees otherwise susceptible to this insect. Unfortunately none of these studies monitored what if any effect these treatments had on the weevil itself. More recent results by Nicole et al. (2006) showed that the biological performance of the weevil was not even slightly affected by such pre-treatments or resin changes (unlike inoculations with the fungus *C. polonica*, Sect. 10.6), however. This again demonstrates that maybe we should expect specialist insects to be able to overcome such 'direct' defences (as discussed in Sects. 10.3.2 and 10.3.3, while also highlighting the limitations of biochemical or genetic studies, if the results are not correlated to function at the ecological level.

It is worth noting that even repeated weevil attacks rarely kill the affected trees, and that the incidence of weevil damage has increased progressively with the introduction of plantation-style forestry practice in North America. It may be that even susceptible tree species have not previously been under selective pressure to evolve any strong resistance to *P. strobi*, and/or would normally succeed in defending themselves from it by indirect means, which have perhaps broken down due to changes in forest management. If further work upholds this as principle cause of *P. strobi* infestations in N. America, then it is less likely that a viable source of natural resistance will be found in native populations. This would reduce the options for controlling this organism to using pesticides, changing forestry practice, or genetic transformation with an exotic defensive trait (see Chap. 11).

Another example of an insect herbivore that has to deal with the resin defenses of conifers is the diprionid pine sawfly *Diprion pini*, which lays eggs on the needles of *P. sylvestris* and *P. nigra*, which the larvae then proceed to eat. Interestingly, the process of egg deposition by *D. pini* females causes an increase in the emission of specific volatiles attractive to *Chrysonotomyia*

ruforum, an egg parasitoid specialized on diprionids living on pines (Hilker et al. 2002a; Mumm et al. 2003, 2004).

This is another example of a tree having a subtle but effective indirect response to an insect herbivore, not unlike the elm system described in Sect. 10.5, and so it will be equally profitable scientifically to study these phenomena in conifers, as with Angiosperm trees of interest. The Max Planck Institute for Chemical Ecology and the Free University in Berlin, are therefore also pursuing a collaborative program to study the molecular genetic responses of *P. sylvestris* to egg deposition by *D. pini* (A. Schmidt, personal communication), in addition to that described for elms.

10.8 Genes and Pathways of Interest

From these previous sections, it should be clear that the list of genes potentially relevant to the defence responses of trees is very large indeed. Here we focus on those pathways associated with inducing the emission of volatiles because of their emerging importance in plant-plant, and plant-insect signalling (Haukioja 2005).

Therefore, rather than attempt to discuss all the possible genes that may be related to the defensive strategies of trees, a brief introduction will be given into the biochemistry of volatile production by plants and the genes that control it, along with some discussion of the current methodologies for studying them. This section will then conclude with a brief discussion as to how these or similar approaches may be utilised for studying other aspects of the defence responses of trees and capturing the genes responsible for further study, including by the use of transformation approaches.

10.8.1 The Biochemistry and Genetics of Plant Volatile Emission

The plant volatiles emitted following herbivore feeding are usually different to those emitted following mechanical or other forms of damage (Dicke et al. 1999; Paré and Tumlinson 1999; Degenhardt et al. 2003; Dudareva et al. 2004; but contrary to Mithöfer et al. 2005). Thus, plants seem to have a broad ability to recognize herbivore attack.

The process of herbivore feeding appears to release specific substances into the tissues of the damaged plant, which elicit direct and indirect defence responses (Turlings et al. 1995; Malone 1996). Plant responses to these stimuli are presumably regulated through a complex network of herbivore or pathogen specific receptors, as well as local and long distance wound signals, the precise combination of which serve to initiate pre-programmed but flexible defence responses (see Sect. 10.4), including insect specific volatile release.

The volatiles reported from herbivore-damaged plants belong to several different chemical classes. One large group are the terpenes (see below), whose structures are all based on the union of C5 isoprenoid units. Another frequently encountered class of volatiles are the so called "green leaf volatiles" (GLVs), which are released very rapidly after damage (Dudareva et al. 2004; D'Auria and Gershenzon 2005).

These six-carbon alcohols, aldehydes, and acetates (Loughrin et al. 1994; Turlings et al. 1998) are products of the lipoxygenase pathway, which begins with the oxidation of linolenic acid, just as in jasmonic acid biosynthesis, although many details of these biosynthetic pathways remain to be elucidated (D'Auria and Gershenzon 2005). Other induced compounds such as methyl salicylate and indole emanate from the shikimic acid/tryptophan pathway (Paré and Tumlinson 1997). It also now appears that in association with the plant hormone ethylene, GLVs may be the responsible agents for plant-plant communication (see Sect. 10.3.3), rather than terpenoids (Ruther and Kleier 2005) – a finding that is bound to increase the interest in characterizing the genes associated with GLV production. These genes seem to exist as large gene families, which raises further questions as to what their ecological or physiological functions may be (D'Auria and Gershenzon 2005).

10.8.2 The Biosynthesis of Terpenoids in Plants

The biosynthesis of terpenoids in higher plants meanwhile is much better understood, and proceeds through two parallel, but probably interconnected pathways (Fig. 10.1; Gershenzon and Kreis 1999; Dudareva et al. 2005), both of which generate the same C5 building blocks for isoprenoid formation, isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). One pathway occurs in the cytoplasm and converts acetyl CoA via mevalonate to IPP (the so-called MVA pathway), while the methyl erythritol (MEP) pathway occurs only in the plastids and uses pyruvate and glyceraldehyde-3-phosphate to generate IPP and DMAPP.

Once formed, IPP and DMAPP units can combine to produce geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20). These linear intermediates are then put through a wide range of cyclizations and rearrangements by individual terpene synthases (encoded by structurally conserved *tps* genes) and cytochrome P450s to produce the huge diversity of mono-(C10), sesqui-(C15), and di-terpene (C20) end products (Dudareva et al. 2004; Martin et al. 2004).

Many genes associated with the biosynthesis of terpenoids in plant species have been cloned and found to be up-regulated after injury and immediately prior to volatile emission begins, or are associated with other aspects of plant-insect signaling, such as scents from flowers (Pichersky and Gershenzon 2002; Degenhardt et al. 2003; Dudareva et al. 2005).

Despite the complexity of terpene biosynthesis, a large number of terpene biosynthetic genes have been isolated from several tree species (Bohlmann et al. 1997; Linden and Phisalaphong 2000; Martin et al. 2003; Arimura et al. 2004). The manipulation of terpene synthases for plant transformation has also been successfully reported on a number of occasions (Spencer et al. 1993; Degenhardt et al. 2003), making it reasonable to consider genetically engineering the terpene composition in any transformable species (Mahmoud and Croteau 2002), including for ecological studies as shown recently for arabidopsis (Kappers et al. 2005; Schnee et al. 2006). Thus, it is likely that the manipulation of tree species for altered terpenoid metabolism and ecological studies will soon follow.

The general approach proceeds as follows: (1) Genes putatively associated with terpene biosynthesis or other volatile emissions are isolated from a cDNA library made from a relevant plant tissue, either by a homology based cloning approach to known terpene biosynthetic genes (Bohlmann et al. 1997; Martin et al. 2004), or increasingly these days after reference to a relevant EST or genomic database. (2) These are then functionally expressed one at a time in a suitable microbial expression system and the activity profile of the encoded protein determine by in vitro assay (Miller et al. 2001; Fäldt et al. 2003b; Martin et al. 2004). (3) Next, it must be decided whether the terpenes whose formation is regulated by this gene are relevant to the ecological interaction under study. (4) Transformation studies then follow, where one attempts to over or under express the gene(s) under study – probably in arabidopsis first as an easy to transform model species, then back into the native host where possible (as advocated by Schnee et al. 2006 with maize). (5) This finally enables ecological studies comparing transformants with wild-type controls. Potentially it should then be possible to study the variation of these genes at the population level, and so determine the selection pressures operating on them, but such studies have yet to be undertaken with forest trees.

Functional expression is necessary for many of the genes associated with the terpenoid pathway, especially terpene synthases (Fig. 10.1), and those associated with other secondary metabolic pathways also, as their precise function cannot be reliably ascertained from their DNA sequence identity alone (Martin et al. 2004). Homology based computer search programs such as Blast™ can give some indication as to a function for many terpene biosynthetic genes but not a definite characterisation. For instance, the very first identification of the important gene responsible for isoprene biosynthesis in poplars (*iso*) – a simple C5 terpenoid emitted mainly in response to abiotic stress (Logan et al. 2000) – was initially confused by it being listed in the EMBL database as a mere limonene synthase (a C10 monoterpene), based on sequence similarity (Miller et al. 2001).

It should be noted, however, that plant terpene formation can be manipulated by other means than just transformation; inhibitors are available that block the synthesis of terpenoids in the two different pathways, so allowing classical physiological approaches to experimentation (e.g. Dudareva et al.

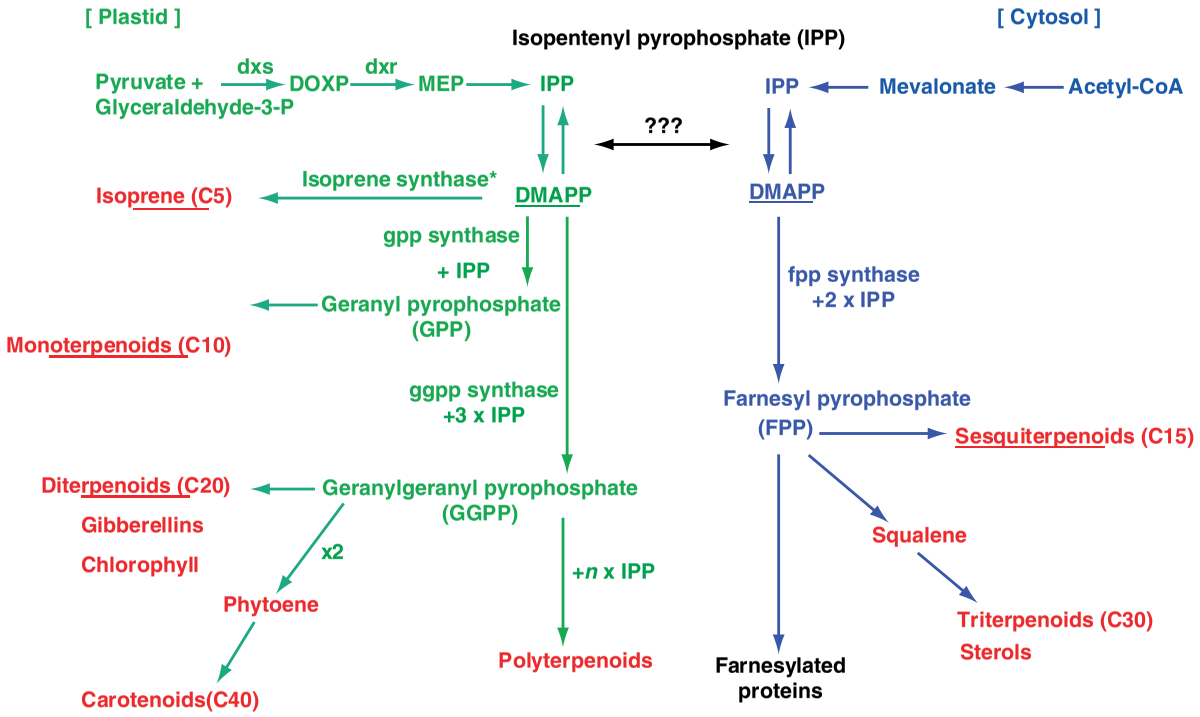

Fig. 10.1. A general scheme for the biosynthesis of terpenoids in plants. The cytosolic mevalonic acid (MAP) pathway for the biosynthesis of terpenoids is shown in *blue*, while the methylerythritol (MEP) pathway of plastids is shown in *green*. Selected terpenoid end products are shown in *red*. Abbreviations (in alphabetical order): DMAPP – dimethylallyl diphosphate; DOXP – 1-deoxy-D-xylulose 5-phosphate (or DXP); *dxr* – 1-deoxy-D-xylulose 5-phosphate reductoisomerase gene; *dx* – 1-deoxy-D-xylulose 5-phosphate synthase gene; FPP – farnesyl pyrophosphate; GPP – geranyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate; IPP – isopentenyl pyrophosphate, or isopentenyl diphosphate (IDP); MEP – 2-C-methyl-D-erythritol 4-phosphate

2005). Furthermore, Kessler and Baldwin (2001) were able to achieve a ~90% reduction in the insect herbivores establishing on wild tobacco (*N. attenuata*) under field conditions, by painting on to plants selected terpene oils characteristic of insect damaged plants and believed to be attractive to insect predators.

Such approaches should not be viewed as being in competition to the use of biochemical, genetic and plant transformation techniques, however, but rather as complementary methodologies. When studying biological phenomena as complicated and diverse as plant defence and environmental responses seem to be, it is important to seek out the most appropriate techniques for investigating the scientific question at issue, rather than adopt one method and go looking for questions it can be used to answer.

In either case, a vital piece of equipment for any lab wishing to undertake such work is some type of volatile collecting system, which can later be analysed in detail by GC-MS or other techniques as appropriate (Millar and Haynes 1998; Dudareva et al. 2004; Tholl et al. 2006). Although portable GC equipment is now available such as the zNose™ (Electronic Sensor Technology, California), most workers still prefer to trap volatiles from headspace and then analyse them using high precision lab based equipment.

This approach enables changes in the volatile emission profiles of plants under treatment to be closely monitored, as well as tracking changes in the relative concentration of individual volatiles, provided they have been identified by authentic standards previously. The above approach is especially valuable for studying the potentially altered emission profiles of transgenic plants either in comparison to control plant lines, and/or when induced by various elicitors or herbivores, as recently performed with Arabidopsis by Kappers et al. (2005), and hopefully soon for field elms (Sect. 10.5) and maybe other tree species.

10.8.3 Further Approaches for Identifying Other Genes of Interest

The critical question when using transformation approaches to study plant defense responses, is which are the best genes to invest all the necessary effort and time in? Clearly, if one is already interested in a particular pathway or sub-set of genes these will be the ones to use, but this pre-supposes that such an interest has already been established. If one's work is driven by an interest in a scientific question and one wants to know which genes may be relevant to it, then other approaches are needed, since pre-selecting particular genes or pathways to study runs the risk of biasing the outlook of ones project towards what is already known before the work even starts.

Making cDNA libraries from experimental plant material is a good start, but these need to be subjected to some form of analysis to determine the gene expression profiles that they contain. If there are good genomic resources available for the tree species under study (e.g. *Pinus taeda*, *Picea* ssp., *Populus* spp.),

then the cDNA samples can be subjected to micro-array analysis to identify which genes are activated in response to the treatment(s).

However, if this choice is not available, other approaches are needed: These can include a large scale sequencing effort of the cDNA libraries, which is effective but expensive. The construction of a subtractive cDNA library to reduce the sequencing effort runs the risk that differentially expressed families of genes with homologous coding regions may be eliminated, as discussed previously, or with some sort of differential display or cDNA-AFLP analysis, but executing these techniques effectively is tricky and prone to artefacts and still have a heavy sequencing requirement. Similar problems can plague the interpretation of micro-array results, but at least it is easier to repeat the experiments once such a micro-array is established.

Either way, it is still likely that too many 'genes of interest' will be highlighted by these approaches to incorporate them all, so further rounds of micro-array experiments or real-time PCR studies are recommended prior to closing off the list of which genes might be included in the transformation part of the program.

In particular, various compounds such as methyl jasmonate, ethylene, salicylic acid, as well as other elicitors specific to the insects or fungi of interest may be used, if known (Nürnberger and Scheel 2001; Pickett and Poppy 2001; Farmer et al. 2003; Belkhadir et al. 2004). This approach has the advantage that elicitors can be used to stimulate and study plant defense reactions in plants under more controlled conditions than is possible with ecological experiments involving field work with insects. This approach has already been used to study the defense responses of conifers (Martin et al. 2002; Hudgins et al. 2004) and elms (Sect. 10.5).

An extension of this strategy that may prove particularly useful for studying the defense responses and associated gene induction in trees, is to use seedlings or ex-vitro clonal propagules as a source of standardized material suitable for lab studies, e.g. with seedlings of *P. abies* (Kozlowski et al. 1999; Elfstrand et al. 2001; Fossdal et al. 2003; Pervieux et al. 2004), or even tissue cultures themselves including those of *P. abies* (Nagy et al. 2005).

Indeed, cell culture systems have been used to study the synthesis of plant secondary metabolites for many years (Hamill et al. 1986), including for studies into the biosynthesis of terpenoids (Spencer et al. 1993; Mahmoud and Croteau 2002; Ishida 2005). More recently this work has been extended to study plant defense responses at the molecular level after treatment with specific elicitors, e.g. for taxol production (Linden and Phisalaphong 2000), as well as other defense related metabolites with other plant cell culture systems (Cane et al. 2005; Zhao et al. 2005).

Indeed, investigations into the altered responses of transformed cell lines can also begin at the cell culture level including with conifers (Elfstrand et al. 2001; Levée and Séguin 2001), speeding the flow of results and hypothesis testing of a trees defense responses and also possibly reducing the need to regenerate every transgenic cell line.

Because of this, there is every reason to believe that such approaches will be fruitful avenue for defence related gene discovery and characterisation in trees. This may also be a promising approach for the preliminary characterisation of defence related genes, metabolites and signalling compounds or elicitors with the caveat that the defence responses of adult trees may not be fully functional or active in such cell culture systems, so the need to examine and test the full trees responses at some stage still remains.

10.9 Advances in Understanding Tree Diseases from Introduced Novel Defensive Traits

This subject of introducing novel defensive traits into trees has been fully reviewed in Chap. 11, but such work can also give insights into the natural defensive mechanisms of trees. One such example was obtained from the transformation of *Populus* ssp with the *Cry1Ac* toxin gene from *B. thuringiensis* (Bt) by the Chinese group Hu et al. (2001), followed by a field trial between 1994 and 1997 (see Chap. 2). This was clearly an exotic and direct defensive trait that would not in any way exist within the natural population otherwise, but in the field trial with these lines, the trees in the non-transgenic control plot suffered seven to eight times more insect damage to their leaves, as compared to the trees in the largely transgenic plot. Growth data for the trees was not provided, however.

This experiment dramatically demonstrates the insect load that poplar trees bear in this region under normal circumstances, as caused by insects susceptible to the Bt toxin *Cry1Ac* at least. Interestingly, one-third of the trees in the transgenic plot were non-transformed poplar lines, but also showed similarly reduced levels of insect damage, as compared to the same non-transgenic lines in the all non-transgenic control plot.

Although the applied aim of this field trial was simply to see if transgenic poplars could be produced that were effectively resistant to the damaging insect pests present in that region, this result provides more evidence for the point raised in Sect. 10.3.3, that group resistance may be more important to long lived forest trees, than for short lived annual plants.

Although the long term durability of Bt toxins for controlling the insect pests of trees has to be questioned, without a much more detailed evaluation of the risk of resistant phenotypes of the affected insects emerging, for the reasons discussed in Sects. 10.3.2 and 10.3.3, this is nevertheless a very significant finding that might not have been made any other way. It is also conceivable, however, that some such mix of insect resistant Bt and susceptible non-Bt trees might be sufficient to limit the risk of Bt resistant insects emerging, possibly causing the entire plantation to 'fail' (Raffa 1989, 2004; Walter and Fenning 2004), as a variant on the non-transgenic buffer zones planted around Bt cotton and corn fields (Bates et al. 2005).

10.10 Studies with Exotic Diseases

It should be apparent from all that has been said here, that trees are highly attuned and adapted to their environment, and usually survive the biotic and abiotic challenges that they routinely encounter. Although they do occasionally succumb to such attacks and ultimately die of old age as will we all, the fact that they can live for centuries is a testimony to their ability to survive these threats most of the time. However, when trees encounter pathogens or insect herbivores to which they have had no prior evolutionary exposure, perhaps because they have been introduced from some other part of the world or because the trees themselves are transplanted to another region, they may not have the means to deal with the attacker.

In extreme cases, introduced pests or diseases have ravaged entire populations of susceptible tree species. Famous examples include Dutch elm disease (DED), caused by the fungus *Ophiostoma novo-ulmi* (Brasier 1991), and chestnut blight caused by the fungus *Cryphonectria parasitica* (Anagnostakis 1995), which were probably transferred to Europe and N. America from East Asia (Brasier 2001). Another example of concern is 'sudden oak death', cause by the fungus *Phytophthora ramorum*, which like *O. novo-ulmi*, is probably a hybrid of two or more mild fungal pathogens from different regions of the world, that were brought into contact by human actions (Brasier et al. 1999; Brasier 2001).

Examples of introduced insect pests causing concern include the chestnut leaf miner *Cameraria ohridella*, which is currently spreading through populations of the European horse chestnut (*Aesculus hippocastanum*), which was probably introduced to Europe from China via Albania in the 1980s (Nardini et al. 2004), and the Asian longhorned beetle (*Anoplophora glabripennis*) which mainly attacks poplar species and may have been spread from China to Europe and North America in wooden packing cases (Cavey 1998).

Serious disease outbreaks due to introduced exotic organisms such as these are mercifully rare, probably due to the extensive generalized 'direct' defenses of trees (Sects. 10.3.1 and 10.3.2), together with the specific feeding or host recognition requirements of many insects and pathogens. However, the chances that insects or pathogens will be able to attack a new host species are probably greater if it is related to their native host, especially if the insects or pathogens are freed from the attentions of their predators and parasites. The occasional severity of these problems highlights the vulnerability of trees to biotic threats when their defenses are either lacking or ineffective, and how effective they are normally.

When specific predators or parasites for the offending organisms cannot be found, and/or any natural resistance which could enable a recovery of the affected population of trees is limited to non-existent, then transformation approaches to introduce additional resistance traits are a reasonable option, so far as political or environmental objections allow (Fenning and Gershenzon 2002; Walter and Fenning 2004; Chap. 11).

There is not the space to include all the examples of transgenic approaches for introducing novel disease resistance traits into trees, but some further discussion of it has already been given in Sect. 10.9 and has been reviewed elsewhere (Peña and Séguin 2001; Gartland et al. 2003; Chap. 11). However, the strategy adopted for transforming elms for resistance to DED is instructive. This began with developing the appropriate tissue culture procedures for regenerating the species after transformation with marker genes (Gartland et al. 2000), and currently includes efforts to modify the physical properties of the tree and its wood to render it less susceptible to the fungus (Gartland et al. 2001), as well as introducing novel genes for anti-fungal peptides, which have already been found to be effective against *O. novo-ulmi* under in vitro conditions (K. Gartland, personal communication). A similar strategy has also being adopted for the American chestnut (Polin et al. 2006).

A further development of this approach that may yield interesting results would be to compare the gene expression profiles of elms or American chestnuts after infection by these introduced diseases to other closely related native pathogens. Given the rapid progress in unraveling the wound perception and signaling pathways in plants (Sect. 10.4), it might thus be possible to identify some failure in these processes in the affected tree species to the introduced diseases. Once identified, the genes responsible for the defective part of the signaling pathway could then be corrected as previously advocated for crop plants (McDowell and Woffenden 2003).

This approach would have the advantage that the engineered resistance to these introduced diseases would be more robustly based, than if 'direct' disease resistance traits alone were used, as well as possibly easing concerns about the effects that introduced novel traits might have on non-target organisms.

10.11 Conclusions

The reason for the detailed description of these plant defence and ecological relationships has been to highlight how attuned trees are to their natural environment, and the care that is needed to understand how their defenses function at the molecular-genetic levels.

The advantage of looking at gene expression levels, rather than biochemical or proteomic changes alone, is that it is usually easier to reliably and consistently quantify changes in gene expression levels, which can indicate that subtle phenotypic changes are the result of an active 'decision' by the plant, rather than due to random sample variation.

However, as powerful as these approaches are, they generate only correlative data about gene function, and can even actively mislead if the phenotype under study is not regulated at the gene expression level, or if some critical

change in gene expression falls below the detection threshold of the DNA micro-array being used.

The expression profiles of selected individual genes can be studied in greater detail by the use of real-time PCR on cDNA produced from plant RNA samples collected at various time points during experimental treatments, but ultimately transforming the native genes under study back into their host in modified form provides the best and most reliable indication of gene function. This is always so, but is particularly important for genes that function at the ecological level, as it may not be possible to ascertain their role otherwise.

Over-expressing a gene of interest in its native host by the use of a constitutive promoter such as 35S is one approach for studying gene function in plants that is also relevant to studying secondary metabolic or defence/environmentally related pathways. However, the products of these biochemical or signalling pathways can be as debilitating to plant tissues as to their herbivores, so inducible expression systems should also be considered (Gatz and Lenk 1998; Padidam 2003; Tang et al. 2004), provided they can be activated without inducing wound or defence pathways, as this might confuse the result.

Of particular value to studies of plant defence or environmentally sensitive responses, is the development of RNA silencing plant transformation cassettes (Elbashir et al. 2001; Wesley et al. 2001; Wang and Waterhouse 2002), as this approach allows the effects of suppressing a native gene to be compared to over-expressing it. Although this approach has not yet been used to study the environmental responses of trees, it will be particularly useful for revealing changes that might affect the behaviour of insect herbivores or predators by particular plant responses that are difficult to tackle by other means.

Although the prospects for conducting genome scale investigations into the defence and environmental responses of trees are improving rapidly, the efficiency, speed and broad applicability of transformation techniques as they apply to trees has not kept pace. "High throughput" is not yet a phrase which can be applied to the genetic transformation of even most annual plants, with the possible exception of the *Agrobacterium*-dip methods used with arabidopsis, so this is clearly a limiting factor when deciding whether and when to use the transformation approach with trees.

Since the ecological and defence phenomena of scientific interest are likely to be very specific to each tree species (as with elms, Sect. 10.5), it will not be possible to transform most of them with more than a few relevant genes in anything resembling a reasonable time frame. Thus, considerable care has to be exercised in the choice of those genes.

Some labs may have a particular interest in one biosynthetic pathway, and so may choose to focus on genes associated with that pathway, but if one is interested in the wider set of responses of a tree to a particular environmental stimulus, then a larger set of choices is likely to present itself. It is important therefore to identify those genes of critical interest that cannot be studied adequately other than by transformation back into the native host.

It is possible to study first the function of many plant secondary metabolic genes in *E. coli* for instance. Those of continuing interest can then be introduced into arabidopsis, in order to determine how they behave in a model plant system (Sect. 10.8.2). This approach should greatly simplify the difficult choices that need to be made as to which genes to transform back into your chosen tree species, and in what permutations to transform them.

Nevertheless, the choice of genes could be simplified even more if the responses of trees to disease, herbivores, environmental stresses and their ecological interactions in general, were studied in greater depth in a few model tree species for which abundant genomic data and efficient transformation procedures are becoming available. A poplar species (but not a hybrid) and a member of the Pinaceae are the most obvious candidates for this effort.

The concerted effort to disentangle the complexities of lignin synthesis and wood formation provides a good model to follow, as many of the genes and sub-pathways involved were studied first in model plant species more amenable for study than trees, and only when some insight had already been gained into the biochemical pathways and genes involved, were these ideas re-tested by transforming the genes identified to be of most interest back into trees, and further tested in scientifically controlled field trials (Li et al. 2003).

Such field trials will be even more essential for studying the responses of trees to diseases, herbivores, environmental stresses and their ecological interactions, as it is not be possible to fully re-create natural conditions under artificial containment, even without considering the size that adult trees can reach. Furthermore, the existence of a set of field trials of transformed poplar and spruce or pines, over and under expressing as large a compliment of environmentally sensitive genes as possible, could serve as an experimental resource for the scientific community as a whole.

It is to be hoped that the environmental and forest scientific community is capable of organising and implementing such an ambitious but achievable scheme.

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11 Fungal and Bacterial Resistance in Transgenic Trees

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11.1 Introduction

In our global economy, the movement of living materials around the world is having a homogenizing effect on previously isolated ecosystems. One of the consequences of this movement is the introduction of many exotic species including plant pathogens. Pathogens that have co-evolved with their host usually reach a balance where both organisms can survive within an ecosystem. But when these same pathogens are introduced to similar species in a different area of the world, the consequences can be devastating. Tree diseases such as white pine blister rust, chestnut blight, beech scale complex, Dutch-elm disease, butternut canker, and dogwood anthracnose, to name just a few examples, have caused significant losses of trees in North American forests and urban settings. Although regulatory safeguards on trade are in place, diseases such as the newly discovered sudden oak death in the western United States, sometimes get through. It is likely that new introductions of diseases and pests will continue into the foreseeable future. In addition, high intensity forest plantations are being developed to supply the world's need for wood products and fiber, which will likely bring new challenges to disease control. Therefore, the development of trees with enhanced resistance to pathogens is necessary to maintain healthy natural forests as well as agroforestry plantations. A transgenic approach for enhancing pathogen resistance in trees is a promising way to restore trees to the forest that were previously devastated by exotic pathogens as well as to prevent such problems in the future.

Significant progress to enhance pathogen resistance through genetic engineering is being made in crop plants. The general approaches include the expression of a variety of antimicrobial proteins or organic molecules, enhancement of the hypersensitive response (HR), and the enhancement of systemic acquired resistance (SAR) (Grover and Gowthaman 2003). Some of the approaches used in crop plants may be applicable to trees, but there are several unique properties of trees to consider when mapping one's disease enhancing approach. These include the long lifespan of a tree, that trees are present through all seasons and might have varying susceptibility among the seasons, and that trees have important associations with symbiotic microorganisms such as mycorrhizae.

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We will cover in this review approaches for enhancing resistance to fungal and bacterial pathogens in woody plant species. In some cases, the resistance enhancing approach will work for only one type of pathogen but in other cases the same or similar strategy would work for both bacterial and fungal pathogens.

11.2 Review of Current Approaches

11.2.1 Chitinases

One of the common approaches to enhancing fungal resistance in transgenic crop plants is to use transgenes encoding fungal cell wall degrading enzymes such as chitinases. This approach has also been used in woody plant species. One of the early and possibly most studied trees transformed with a transgenic chitinase was apple (*Malus* × *domestica*). In the first study, two of three transgenic lines of 'Royal Gala' constitutively expressing an endochitinase from *Trichoderma harzianum* exhibited increased resistance to apple scab caused by *Venturia inaequalis* (Wong et al. 1998). Similarly, in another experiment, six out of eight transgenic lines of 'Marshall McIntosh' showed reduced symptoms of apple scab and the symptoms were negatively correlated with endochitinase expression (Bolar et al. 2000). Interestingly, these authors also noted that vigor was lower in the transgenic trees and that the growth was also negatively correlated to the level of endochitinase expression. In a third study, 'Marshall McIntosh' apple was transformed with an endochitinase and an exochitinase from *Trichoderma atroviride*, singly and in combination (Bolar et al. 2001). Apple scab resistance was correlated with the levels of expression of either enzyme, but the exochitinase was less effective than the endochitinase. As with the previous work, endochitinase expression was negatively correlated to growth, but the exochitinase had no consistent effect on plant growth. Plants transformed with both genes demonstrated higher levels of resistance indicating that the enzymes act synergistically. In a fourth study, two different cultivars of apple ('Galaxy' and 'Ariane') were transformed with the same exochitinase and endochitinase genes (Faize et al. 2003). The transgenic plants showed enhanced resistance to apple scab and exhibited a reduced growth. The reduced growth appeared to be associated with increased lignin content, and increased peroxidase and glucanase activities. All of these studies in apple used either the CaMV 35S promoter or enhanced CaMV 35S promoter that can produce high levels of expression in most tissues. It would be interesting to evaluate whether a more controlled expression of the endochitinase using inducible or tissue specific promoters could maintain the resistance enhancing ability of this enzyme while reducing the detrimental effect on plant growth.

Transgenic grapevines, although not trees, were also one of the first woody plant species expressing a transgenic chitinase. In one example *Vitis vinifera*

L. cv. 'Neo Muscut' was transformed with a constitutively expressed rice class I chitinase gene via *Agrobacterium*-mediated transformation (Yamamoto et al. 2000). Two of the 20 independent transgenic plant lines showed enhanced resistance to powdery mildew caused by *Uncinula necator* in greenhouse tests. In addition, these transgenic plants showed slightly enhanced resistance to *Elisinoe ampelina* inducing anthracnose. However, field trials were not reported.

A recent study (Noël et al. 2005) also shows successful introduction and expression of the endochitinase *ech42* cDNA from *T. harzianum* in two different woody plant species, black spruce (*Picea mariana*) and hybrid poplar (*P. nigra* × *P. maximowiczii*). Several lines of transgenic spruce showed an increased resistance to the root rot disease causal agent *Cylindrocladium floridanum*. In vitro pathogenesis tests also demonstrated that the transgenic poplars had increased resistance to *Melampsora medusae* leaf rust. These results show that constitutive expression of an endochitinase gene could be exploited to enhance resistance to fungal pathogens in important forest tree species without detrimental effects on plant growth.

Silver birch (*Betula pendula*) was transformed using a constitutively expressed chitinase IV from sugarbeet via *Agrobacterium*-mediated transformation (Pappinen et al. 2002). The resulting transgenic lines with the highest levels of expression showed the highest levels of resistance to the leaf spot fungus *Pyrenopeziza betulicola* in greenhouse tests. A three-year field trial was subsequently carried out and gave different results. The transgenic birch lines retained their various levels of chitinase IV expression, but the high levels of expression did not significantly improve resistance to natural infections of the *P. pendula* leaf spot disease (Pasonen et al. 2004). The authors did report that these transgenic birch lines showed an "improving effect on most parameters of birch rust" caused by *Melampsoridium betulinum*. This research is a good example of the importance of field trials when assessing disease resistance.

11.2.2 Antimicrobial Peptides

11.2.2.1 Short Amphipathic Cationic Peptides

Small, cationic antimicrobial peptides are commonly found in organisms as part of their natural defense system against pathogens (Hancock and Diamond 2000). For example, the magainins consist of 21–26 amino acid residues that are strongly basic which are expressed in the skin and intestines of frogs (Zasloff 1987). These natural peptides exhibit bacteriocidal, fungicidal and virucidal activities (Zasloff 1987; Aboudy et al. 1994). Their cytotoxic effect results from a disruption of the electrochemical gradient across free-energy transducing membranes (Bechinger 1997). Cecropins are short (20–40 amino acids) amphipathic peptides that were isolated from the

haemolymph of bacteria challenged *Lepidoptera* and *Diptera* (Bulet et al. 2004). They are effective against Gram-positive and Gram-negative bacteria that cause disease in plants and mammals (Kadono-Okuda et al. 1995; Giacometti et al. 1998). These antimicrobial peptides act by producing channels of various sizes which affect the integrity of lipid bilayers which has been suggested to be the main reason for the cytotoxic effect of these polypeptides (Bechinger 1997). T4 lysozyme (T4L) plays a major role in the lytic cycle of bacteriophage T4 by releasing virions from the dying bacterial host (Poteete and Hardy 1994). T4L was originally thought to possess bactericidal activity through its capacity to hydrolyse the murein layer in bacterial cell wall (During 1996). However, later studies showed that the C-terminal amphipathic region present in T4L and egg white lysozyme showed antimicrobial activity even when the enzymes were heat denatured to abolish their hydrolytic activity (During et al. 1999). Therefore, the most likely mechanism for the antimicrobial activity of T4L is disruption of lipid membranes similar to what is observed with cecropins and magainins. Because many of these antimicrobial peptides are relatively non-toxic to multicellular organisms but can eliminate or inhibit the growth of a wide range of bacteria, fungi, and protozoa, they are being tested in a variety of applications. For example, transgenic mice expressing a cecropin-class lytic peptide showed enhanced resistance to *Brucella abortus* (Reed et al. 1997). Antimicrobial peptides are also finding uses in transgenic trees.

Because of an antimicrobial peptide's small size and simple structure, synthetic analogs can be developed and tested for changes in activity. Synthetic derivatives of natural peptides have been shown to be efficient antimicrobial agents against a broad spectrum of plant and animal pathogens (Schwab et al. 1999; Tossi et al. 2000; Rajasekaran et al. 2001; Ballweber et al. 2002). We have tested amphipathic, cationic peptide designs based generally on the three-dimensional structure and positive charge vs hydrophobic distributions of magainins (Zasloff 1987), yet with totally unique amino acid sequences (Powell et al. 1995). Because the peptides were designed from scratch, the number of highly hydrophobic amino acids could be reduced to minimize hemolytic effects while maximizing antifungal activity. Many proteinase recognition sites were also added to the amino acid sequence so that the peptides would be easily and quickly digested to help reduce any possibility of allergic reactions due to ingestion. Synthetic peptides that were effective at inhibiting the growth of the chestnut blight fungus, *Cryphonectria parasitica*, as well as other fungi and bacteria and at the same time have a negligible effect on humane red blood cells and pollen (Powell et al. 1995, 2000; Powell and Maynard 1997) were then reverse engineered into synthetic genes.

Liang et al. (2002) produced transgenic hybrid poplars carrying a construct expressing ESF12, one of the synthetic antimicrobial peptide designs, and Ac-AMP1.2, an analog of the cystein-rich, antimicrobial peptide Ac-AMP1 (Broekaert et al. 1992) from *Amaranthus caudatus*, under control of a

wound-inducible promoter, *win*3 (Hollick and Gordon 1993, 1995) from poplar. Using leaf disk assays, transgenic poplar showed resistance to *Septoria musiva*. The ESF12 and Ac-AMP1.2 coding regions were placed adjacent to one another to encourage normal expression of the ESF12 peptide and leaky expression (Skuzeski et al. 1990, 1991) of the Ac-AMP1.2 peptide. In the same research a construct constitutively expressing the Ac-AMP1.2 peptide also enhanced pathogen resistance to *Septoria* leaf spot (Liang et al. 2002).

In initial field trials, these transgenic hybrid poplars also showed enhanced resistance to *Septoria* cankers (Powell, Liang, and Maynard, unpublished) using an inoculation method on young, green stems (Weiland et al. 2003). There was no significant difference in growth between the transgenic trees and the non-transgenic controls during the first year. These tests are currently being repeated on two-year-old stems to determine if the resistance is maintained in these older tissues.

Another synthetic peptide, D4E1, was expressed in poplar to high levels and the transgenic lines were less susceptible to two commercially important bacterial pathogens, *Xanthomonas populi* and *Agrobacterium tumefaciens* (Mentag et al. 2003). Cecropins synthetic analogs were also introduced in apple (Norelli et al. 1998). Although some of the transgenic lines showed an improved resistance to fire blight caused by *Erwinia amylovora* in field trials, the cecropin-expressing lines were not statistically different from the vector control lines.

11.2.2.2 Cystein-rich Peptides

Most antimicrobial peptides from plants fall in this category. They are divided into thionins, defensins, lipid transfer proteins (LTP), heveins and knottins (Broekaert et al. 1997). They are well known for their ability to inhibit the growth of fungi and bacteria in vitro. Their mode of action is not yet well understood and the majority of them do not cause ion leakage as observed with amphipathic peptides. A common biological activity among cystein-rich peptides is the inhibition of enzymes through direct peptide-protein interactions (Broekaert et al. 1997).

The apple cultivar 'Jonagold' was transformed with two types of cys-rich antimicrobial peptide transgenes, both under the control of the CaMV 35S promoter (Bondt et al. 1998). Protein extracts from the transgenic trees expressing Rs-AFP2, a defensin-like antimicrobial peptide from radish, showed a 32-fold increase in fungal growth inhibition compared to extracts of control plants. Extracts from the transgenic trees expressing Ace-AMP1, an LTP antimicrobial peptide from onion show a fourfold increase in fungal growth inhibition compared to controls. To date, further bioassays on whole plants have not been published.

In another study, two apple genotypes were transformed with a transgene encoding a wheat purindoline-b (PinB), a cysteine-rich antifungal peptide of

the LTP family (Faize et al. 2004). One apple genotype, 'Ariane,' carried a natural resistance *Vf* gene while the other apple genotype, 'Galaxy,' did not. The transgenic apples were challenged with two different races of the fungal pathogen, *V. inaequalis*: race 6 which could overcome the *Vf* resistance and race 1 which could not. A significant negative correlation between PinB content and susceptibility was observed in both transgenic genotypes when challenged with race 6. This correlation was not seen in 'Galaxy' when inoculated with race 1. This research shows that different host/pathogen interactions can influence the effectiveness of the PinB transgene.

11.2.2.3 Attacins

Attacins are antimicrobial proteins that are synthesized in giant silk moth, *Hyalophora cecropia*, pupae haemolymph in response to bacterial infection. They differ from the other classes of antimicrobial peptides by their larger size (20 kDa) but, like amphipathic peptides, cause alterations in the structure and permeability properties of the bacterial outer membrane (Engstrom et al. 1984). These alterations are associated with a specific inhibition of the synthesis of outer membranes proteins (Carlsson et al. 1991, 1998). The *attE* gene, encoding an attacin, has been successfully introduced into the genome of apple and pear to improve the resistance of these species to fire blight. Partial resistance was obtained in transgenic lines expressing attacin in the cytoplasm (Norelli et al. 1994; Reynoird et al. 1999). More resistance was achieved by forcing the secretion of attacin into the intercellular space, which exposed the pathogen to the antimicrobial agent at an earlier stage of infection (Ko et al. 2000). In a later study, the same research group noted an absence of synergy between attacin and T4L (Ko et al. 2002). Since both peptides alter the integrity of the bacterial membrane, this result is not surprising.

Antimicrobial peptides have also been isolated from trees and they might be used to engineer microbial resistance. For example, the white spruce defensin pgD1 caused extensive growth inhibition of three fungal pathogens in vitro (Pervieux et al. 2004). Another example is a polypeptide isolated from seeds of the tropical tree *Moringa* sp. that displays bacteriostatic and bactericidal activities against several Gram-positive and Gram-negative bacteria (Suarez et al. 2003). These peptides illustrate that trees, like other plants, have active defense mechanisms and that the next generation of transgenic trees could use endogenous genes with altered expression patterns.

11.2.3 Oxalate Oxidase

Oxalate oxidase (Lane et al. 1986, 1993; Lane 1994; Dumas et al. 1995) is an interesting and potentially useful enzyme because it converts oxalate into H_2O_2 and CO_2. Therefore, this enzyme could be involved in several resistance-

enhancing mechanisms. First, it may simply remove the oxalic acid produced by the fungus. We have shown that in the presence of oxalic acid, lignin formation in American chestnut callus is inhibited while transgenic callus expressing oxalate oxidase forms lignin in normal amounts (Welch, Stipanovic, Maynard, and Powell, unpublished). This effect could be caused merely by the degradation of the oxalic acid. But there is also the possibility that the H_2O_2 byproduct could contribute to peroxidase-catalyzed reactions during lignification of cell walls (Olson and Varner 1993; Thordal-Christensen et al. 1997). The H_2O_2 byproduct might also have a direct antimicrobial effect or possibly signal other defense mechanisms.

The 'Ogy' clone of hybrid poplar has been transformed with a transgene encoding a wheat oxalate oxidase under control of the CaMV 35S promoter. The resulting trees showed significant resistance to *Septoria musiva* in leaf disk assays (Liang et al. 2001). In initial field trials, these transgenic trees also showed enhanced resistance to *Septoria* cankers (Powell, Liang, and Maynard, unpublished) using an inoculation method on young, green stems (Weiland et al. 2003). These tests are currently being repeated on two-year-old trees. In the field trials of the transgenic hybrid poplar constitutively expressing the oxalate oxidase gene, it was observed that the transgenic trees grew significantly slower than the non-transgenic controls and transgenic poplar expressing antimicrobial peptides (Powell, Liang, and Maynard, unpublished). The reason for this phenomenon in the field trials is not known, but it might be caused by a reduction in oxalic acid production by the plant itself which could inhibit phosphorus uptake in the roots (Strom et al. 2002). Further studies are needed to determine the cause of the slower growth, but the effect might be avoided by using a regulated promoter to drive the expression of the oxalate oxidase gene.

11.2.4 RNA Interference (RNAi or Post-transcriptional Gene Silencing [PTGS])

Agrobacterium is currently being used as a biological vector for transformation of plants, fungi and animals (Gelvin 2003). This capacity of *Agrobacterium* to achieve inter-kingdom transfer of DNA into its host is a natural pathogenicity mechanism. To cause disease, *Agrobacterium* must first deliver tumorigenic DNA (T-DNA) into the plant genome. After integration, the T-DNA promotes the synthesis of nutritive compounds that provide a selective advantage for *Agrobacterium* (Escobar and Dandekar 2003). Three genes are in the T-DNA that encodes a tryptophan monooxygenase (*iaaM*), an indole-3-acetamide hydrolase (*iaaH*) and an AMP isopentyl transferase (*ipt*). These genes cause hormonal deregulations in the infected tissue, which is responsible for the appearance of tumours. Their removal from the T-DNA will result in the absence of gall formation (Ream et al. 1983). These results prompted two independent research groups in the development of a transgenic strategy based on

RNA interference (RNAi) to block the accumulation of *iaaM* or *ipt* transcripts and therefore prevent crown gall disease (Escobar et al. 2001; Lee et al. 2003).

RNA interference is a mechanism that targets specific RNAs for degradation. The targeting is based on sequence identity and can be initiated in diverse ways (reviewed in Dykxhoorn et al. 2003). Generally, RNAi is initiated through the detection of double-stranded RNA by a complex called Dicer. This complex will cleave the double-stranded RNA into short-interfering RNAs (siRNA). In turn, these siRNA will be recognized by the RISC complex and together they will initiate the cleavage of the target RNA (reviewed in Voinnet 2002). The Nature Publishing Group as a web focus that contains a selection of published reviews, perspectives and highlights on RNAi and a helpful animation on the mechanics of RNA interference (http://www.nature.com/focus/rnai/index.html).

A simple way to initiate RNAi artificially is to transfer a construct to the plant genome that will generate double-stranded RNA. With that in mind, both groups designed constructs that would give rise to *iaaM* and *ipt* self-complementary RNA and initiate RNAi against these oncogenic genes. After successfully blocking *Agrobacterium* tumorigenesis in *Arabidopsis,* tomato and tobacco (Escobar et al. 2001; Lee et al. 2003), the investigators tried their strategy in walnut and apple. A high percentage of self-complementary RNA expressing walnut lines showed improved resistance to *Agrobacterium* while antisense silencing was not successful (Escobar et al. 2002). In the RNAi expressing lines, the average tumour mass was reduced by one to two orders of magnitude when compared to the wild type untransformed lines. A similar strategy was very efficient in preventing gall formation on apple roots, the organ most affected by crown gall in the field (Viss et al. 2003).

Studies on gene silencing have shown that RNAi was transmissible through grafting but not transmissible to the next generation (Palauqui et al. 1997; Voinnet et al. 1998; Sonoda and Nishiguchi 2000). This is an attractive feature because scions could be grafted to silenced rootstock generating stems, flowers and fruits that would be resistant to crown gall without being transgenic. However, experiments with tobacco and tomato did not show transmissibility of the oncogene silencing and crown gall resistance through grafts (Escobar et al. 2003; Lee et al. 2003). A recent study has shown that the target RNA must be present for the systemic spread of the silencing signal (Garcia-Perez et al. 2004), so it is not surprising that oncogene silencing was not transmissible to wild type plants through grafts.

11.2.5 Plantibodies

Another way to counteract pathogenic genes is to block the activity of the resulting proteins. This can be achieved through the production of recombinant antibody fragments. Recombinant antibodies directed against the coat protein of viral pathogens were used successfully to prevent viral

multiplication (Ziegler and Torrance 2002; see Chap. 9). Using a similar approach, Le Gall et al. (1998) engineered tobacco plants to express a recombinant antibody fragment directed against the major membrane protein of stolbur phytoplasma. Transgenic shoots grafted on infected rootstocks remained symptomless while untransformed grafts showed all the symptoms of infection.

The first step in designing an antibody that will protect the plant is to identify a protein that is essential for pathogenesis and is available to the plant to be inactivated. In many plant-microbial interactions, proteins are delivered by the pathogen to the host cytoplasm via complex secretion systems (Gauthier et al. 2003). The functions of delivered proteins include suppression of defense mechanisms, modification of the host signal transduction pathway, degradation of host proteins and promotion of lesion formation (Alfano and Collmer 2004). Identification of effector proteins is a key process in order to develop an efficient strategy based on recombinant antibodies.

Citrus canker is caused by the bacterial pathogen *Xanthomonas citri* (syn. *Xanthomonas campestris* pv. *citri* or related *Xanthomonas campestris* pv. *aurantifolii*). It is a serious disease of most commercial citrus cultivars and some citrus relatives (Schubert et al. 2001). Citrus canker is characterized by the formation of circular, water soaked lesions that become raised, growing into white or yellow spongy pustules that eventually darken and thicken into a brown corky canker which is rough to the touch (Brunings and Gabriel 2003). An effector protein, PthA, normally injected into plant cells via a Type III secretion system has been identified in *X. citri* (Yang and Gabriel 1995). Upon delivery into the host cells, PthA is imported into the nuclei where it might interact with the transcriptional machinery through heptad repeats similar to leucine zippers (Yang and Gabriel 1995). Its presence in citrus cells elicits division, enlargement and death of host cells, which are symptoms associated with the first stages of canker formation (Duan et al. 1999). Researchers at Integrated Plant Genetics (IPG, http://www.ipgenetics.com/) have developed the Disease Block™ technology, which consists in the expression in transgenic citrus of a recombinant antibody fragment directed against PthA. The cytoplasm expressed antibody fragment should bind to PthA upon delivery into the host cell and prevent its nuclear localisation. The strategy did not produce fully resistant plants but was efficient in slowing the disease process that resulted in a 2000-fold reduction in the number of *X. citri* cells released after one cycle of infection. Complete immunity is considered possible by improving the stability and expression level of the recombinant antibody.

11.2.6 Other Resistance-enhancing Transgenes

A few other examples of enhancing pathogen-resistance in tree species include using the Ri-plasmid from *Agrobacterium* to transform English elm (*Ulmus procera*) in an effort to alter elm xylem structure and therefore

enhance resistance to Dutch-elm disease (Gartland et al. 2001). This research is still ongoing. The apple cultivar "Gala" was transformed with natural resistance gene, *HcrVF2*, from wild apple (*Malus floribunda*) and four independent transgenic lines showed resistance to apple scab caused by *V. inaequalis* (Belfanti et al. 2004).

Not all transgenes tested have demonstrated an ability to enhance pathogen resistance. Often these tests never are published, but they are informative. For example, the bacterio-opsin (*bO*) that had previously been shown to induce hypersensitive-response-like lesions, increase viral and bacterial disease resistance, and stimulate pathogenesis-related gene expression in tobacco did not enhance fungal resistance in transgenic hybrid poplar clones (Mohamed et al. 2001). More recently, Giorcelli et al. (2004) engineered white poplar with a stilbene synthase gene from grape. Transgenic poplars produced resveratrol glucosides, which have previously been shown to increase fungal resistance in transgenic alfalfa (Hipskind and Paiva 2000). Despite the presence of high amounts of resveratrol glucosides, in vitro bioassays revealed no increase resistance against the pathogen, *Melampsora pulcherrima*. Therefore, although some transgenes appear to be promising resistance-enhancing genes in herbaceous plants, not all of them will prove to be as useful in woody species.

11.3 Next Steps

From the research described above, as well as from the abundance of research completed with herbaceous crop species, at least four conclusions can be made. First, it is possible to enhance a tree's resistance to a given pathogen or pathogens. Second, the genotype of the starting material might influence the effectiveness of the chosen strategy. Third, combinations of transgenes, i.e. gene pyramiding, tend to give higher levels of resistance than when the genes are used singularly. Logically this should also increase the durability or sustainability of the resistance, although this has not been directly tested. Fourth, constitutive expression of many of these transgenes can reduce the growth rate of the plant. Therefore, future research should take these results into consideration.

Two studies performed on trees reported differential efficacy in different host/pathogen interactions. First, the overexpression of *PinB* was more efficient in an apple clone that carried a natural resistance gene (see above). We also observed a better protection in a chitinase overexpressing line against poplar leaf rust when a natural resistance is present (Boyle et al. 2005). In this study, clone NM6 carries a partial resistance to *Melampsora medusae* but it is fully susceptible to *M. larici-populina*. The amount of *M. larici-populina* was reduced by half after a complete growth cycle on the chitinase overexpressing line compared to the wild type plant. However, this is not sufficient because

the pathogen is still able to produce urediniospores and spread. In contrast, chitinase overexpression not only blocked the growth of *M. medusae* but also caused a reduction of pathogen number 14 days post-inoculation. It was so efficient that the infection remained symptomless, similar to what is normally observed in a non-host reaction. Therefore, it might be better to build on existing natural defenses than to rely only on single transgene expression.

In our research we are exploring three ways to deliver multiple transgene products. The obvious way to accomplish this is to place multiple transgene constructs on a single vector. The use of this strategy can be limiting because the larger the T-DNA region grows, the more difficult subcloning becomes and the efficiency of transformation can also be reduced (Park et al. 2000). This could be problematic with tree species because often the transformation and whole-plant regeneration procedures are not fully optimized and therefore give low transformation rates. One way to circumvent this problem is to perform cotransformation with several smaller vectors (Halpin et al. 2001; Laigeng et al. 2003). We have accomplished this with transformation of American chestnut somatic embryos (Powell, unpublished) and white spruce embryogenic tissue (Séguin, unpublished). However, cotransformation does not solve all the problems. When placing multiple transgene constructs into a tree, duplication of sequence should be avoided to reduce the probability of gene silencing (Park et al. 1996; Thierry and Vaucheret 1996; Chareonpornwattana et al. 1999). This requires the use of many different promoters to drive the individual transgene's expression. One way to reduce the number of promoters needed would be to express multiple gene products from a single transgene construct. This can be accomplished by using a self-cleaving protein based on the NIa proteinase from tobacco etch virus (TEV) (Carrington et al. 1988; von Bodman et al. 1995; Ceriani et al. 1998; Dasgupta et al. 1998). We have constructed a transgene containing two resistance-enhancing gene products, an endochitinase (Lorito et al. 1998) and an oxalate oxidase with the NIa proteinase in the middle, connected by cleavage recognition sites (Liang et al. 2005). We chose these two enzymes because both have been shown to enhance pathogen resistance by completely different mechanisms. This construct has been tested in *Arabidopsis* and was shown to cleave the polyprotein into the individual enzymes. We are currently using this construct in our American chestnut transformation experiments.

The fourth point about problems with using a constitutive promoter can be avoided by incorporating regulated promoters into the construct design. Several of these regulated promoters are already available including PAL promoters from poplar (Gray-Mitsumune et al. 1999), poplar wound-inducible promoters (Clarke et al. 1994; Hollick and Gordon 1995), American chestnut vascular promoters (Connors et al. 2002), as well as many from herbaceous plants that might also be useful in tree species. For example, we have shown that a soybean vascular promoter from the *Vsp*B gene (Mason et al. 1993; Sadka et al. 1994) can drive expression of oxalate in transgenic American chestnut shoots (Polin et al. 2005). As the era of genomics progresses, the

Table 11.1. Strategies for enhancing pathogen resistance in transgenic trees

Strategy	Disease	Pathogen	Reference
Antimicrobial peptides			
	Poplar leaf spot and canker	*Septoria musiva* (fungus)	Liang et al. (2002); Powell et al. (unpublished)
	Apple scab	*Venturia inaequalis* (fungus)	Faize et al. (2004)
	Crown gall of poplar	*Agrobacterium* sp (bacteria)	Mentag et al. (2003)
	Poplar canker	*Xanthomonas populi* (bacteria)	Mentag et al. (2003)
	Fire blight of apple and pear	*Erwinia amylovora* (bacteria)	Norelli et al. (1994, 1998); Reynoird et al. (1999); Ko et al. (2000, 2001)
Chitinases			
	Apple scab	*Venturia inaequalis* (fungus)	Wong et al. (1998); Bolar et al. (2000, 2001); Faize et al. (2003)
	Powdery mildew of grapevines	*Uncinula necator* (fungus)	Yamamoto et al. (2000)
	Birch leaf spot (fungus)	*Pyrenopeziza betulicola*	Pappinen et al. (2002)
	Poplar rust	*Melampsora* sp (fungus)	Boyle et al. (2005); Séguin et al. (unpublished)
	Spruce root rot	*Cylindrocladium floridanum* (fungus)	Noël et al. (2005)
Oxalate oxidase			
	Poplar leaf spot and canker	*Septoria musiva* (fungus)	Liang et al. (2001); Powell et al. (unpublished)
Resistance gene			
	Apple scab	*Venturia inaequalis* (fungus)	Belfanti et al. (2004)
RNA interference			
	Crown gall of walnut and apple	*Agrobacterium* sp (bacteria)	Escobar et al. (2003); Viss et al. (2003)
Antibodies			
	Citrus canker	*Xanthomonas citri* (bacteria)	http://www.ipgenetics.com/

availability of promoters is exploding. One can now perform database mining to select a gene with a useful expression pattern, and then use the gene's sequence to clone the adjacent promoter. If it has not been previously characterized, this promoter will need to be connected to a reporter gene to confirm its expression pattern as part of a transgene, but the variety of promoters

becoming available should allow much more controlled expression of resistance-enhancing genes. Use of regulated promoters will help in preventing detrimental effects to the plant and non-target organisms as well as improve the effectiveness of the transgene.

11.4 Conclusions

From this review it is clear that pathogen resistance can be enhanced in transgenic trees using genetic engineering techniques (Table 11.1, Chaps. 10 and 12). Beyond the technical abilities comes the next question, will these trees become widely used? This will largely depend on public acceptance. We need to learn from past mistakes and current successes in GM food research. In order to obtain broad public support, the resistance-enhanced transgenic trees must demonstrate public utility and environmental safety. According to Don S. Doering, today's stakeholder demands more than regulatory compliance and favors products that do "more good" rather than just "less harm" (Doering 2001). Therefore, researchers must not only be technically competent, but must also make connections with the public. The research must be as transparent as possible, even though there is danger from radical groups wishing to stop the research. For example, in field trials for transgenic American chestnut and American elm, educational plots were included in the research design to give the public direct access to a part of the ongoing research. The public must become aware of the benefits of the research through the mass media and through public presentations. The benefits should not be overblown or understated, but factual. Only in this way will the public embrace the use of transgenic trees.

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12 Genetically Modified Trees Expressing Genes for Insect Pest Resistance

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12.1 Introduction

The increasing number of genetically modified (GM) insect resistant plants produced by different public institutions and private companies all over the world originates from the strong efforts made in order to develop a new technology, able to increase yields and quality with limited costs and to provide environmental benefits. The search for novel useful genes encoding insecticidal proteins is still in progress and information deriving from the prolonged cultivation of commercialized GM products confirm the efficacy of this biotechnology application. However, results from field trials performed with transgenic plants suggest an opportunity in adopting integrating pest management (IPM) strategies to obtain a durable and safe protection (Bates et al. 2005).

As for the major crops, insect resistance is considered one of the relevant goals in perennial plant breeding programmes. Insects are responsible for substantial damages in woody plant species, resulting in severe reduction of growth rates and production, which have been estimated in about one-third of the total potential production before harvest (Speight and Wainhouse 1989). At present, plant protection in commercial agricultural systems is based predominantly on the use of synthetic insecticides deleterious to human health and environment. Due to the need of combining the increasing demand for wood and food products to the economical, environmental and social expectations, new molecular approaches need to be explored. Extensive reviews dealing with the different aspects of this topic have been published (James 1997; Walter et al. 1998). However, the use of genetic engineering to improve the insect pest resistance trait of forest and fruit trees has rapidly evolved. New research fields include the use of novel transgenes and improved transformation protocols specifically developed for recalcitrant species and cultivars. The significant advances obtained are reported in the text and summarized in Table 12.1.

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Table 12.1. Overview of transgenes for insect pest resistance that have been transferred into forest and fruit trees

Transgenic plants	Transgenes[a]	Gene transfer technique	Target insects	References
Bt toxins				
Populus alba × *P. grandidentata*	*cryIA(a)*	Biolistics	*Malacosoma disstria*	McCown et al. (1991)
Picea glauca	*cryIA*	Biolistics	*Choristoneura fumiferana*	Ellis et al. (1993)
Malus × *domestica*	*cryIA(c)*	*A. tumefaciens*	–	James et al. (1993)
Populus alba × *P. grandidentata*	*cryIA(a)*	Biolistics	*Malacosoma disstria; Lymantria dispar*	Robison et al. (1994)
Populus nigra	*cryIAc646*	*A. tumefaciens*	*Hyphantria cunea; Lymantria dispar*	Balestrazzi et al. (1994)
Populus alba	*cryIAc646*	*A. tumefaciens*	–	Balestrazzi et al. (1994)
Larix decidua	*Bt-δ endotoxin*	*A. rhizogenes*	*Coleophora varicella*	Shin et al. (1994)
Populus nigra	*cryIA(c)*	*A. tumefaciens*	*Apocheima cinerarius; Lymantria dispar*	Wang et al. (1996)
Juglans regia	*cryIA(c)*	*A. tumefaciens*	*Laspeyresia pomonella*	Dandekar et al. (1998)
Populus trichocarpa × *P. deltoides*	*cryIIIA*	*A. tumefaciens*	*Chrysomela scripta*	Meilan et al. (2000)
Populus × *euramericana*	*cryIIIA*	*A. tumefaciens*	*Chrysomela scripta*	Meilan et al. (2000)
Eucalyptus camaldulensis	*cryIIIA*	*A. tumefaciens*	*Chrysophtharta spp.*	Harcourt et al. (2000)
Populus nigra	*cryIA(c)*	*A. tumefaciens*	*Apocheima cinerarius; Orthosia incerta*	Hu et al. (2001)
Populus tremula × *P. tremuloides*	*cryIIIA(a)*	*A. tumefaciens*	*Chrysomela tremulae*	Génissel et al. (2003)
Pinus taeda	*cryIA(c)*	Biolistics	*Dendrolimus punctatus; Cryptothelea formosicola*	Tang and Tian (2003)
Pinus radiata	*cryIA(c)*	Biolistics	*Teia anartoides*	Grace et al. (2005)

Proteinase inhibitors				
Malus × *domestica*	*CpTi*	*A. tumefaciens*	–	James et al. (1993)
Betula pendula	*pinII*	*Agrobacterium spp.*	–	Pappinen et al. (1995)
Populus tremula × *P. tremuloides*	*OCI*	*A. tumefaciens*	*Chrysomela tremulae*	Leplé et al. (1995)
Populus × *euramericana*	*pinII*	*A. tumefaciens*	*Chrysomela scripta*	Kang et al. (1997)
Populus alba × *P. grandidentata*	*pinII*	*A. tumefaciens*	*Plagiodera versicolora*	Heuchelin et al. (1997)
Populus nigra	*CII; PI IV*	*A. tumefaciens*	–	Confalonieri et al. (1997)
Populus nigra	*KTi3*	*A. tumefaciens*	*Lymantria dispar; Clostera anastomosis*	Confalonieri et al. (1998)
Populus alba	*Sbcys*	*A. tumefaciens*	–	Delledonne et al. (1998)
Populus alba	*Atcys*	*A. tumefaciens*	*Chrysomela populi*	Delledonne et al. (2001)
Malus × *domestica*	*Atcys*	*A. tumefaciens*	*Melolontha melolontha*	Romanò et al. (2004)
Other strategies				
Liquidambar styraciflua	*POD*	*A. tumefaciens*	*Hyphantria cunea; Lymantria dispar*	Dowd et al. (1998)
Citrus paradisi	*gna*	*A. tumefaciens*	–	Yang et al. (2000)
Populus deltoides × *P. simonii*	*AaIT*	*A. tumefaciens*	*Lymantria dispar*	Wu et al. (2000)
Malus × *domestica*	*Avd*	*A. tumefaciens*	*Epiphyas postvittana*	Markwick et al. (2003)
Malus × *domestica*	*Stv*	*A. tumefaciens*	*Epiphyas postvittana*	Markwick et al. (2003)
Populus tremula × *P. alba*	*TDC1*	*Agrobacterium spp.*	*Malacosoma disstria*	Gill et al. (2003)
Populus tremula × *P. alba*	*PtdPPO*	*A. tumefaciens*	*Malacosoma disstria*	Wang and Constabel (2004)

[a] *AaIT*: scorpion neurotoxin gene; *Atcys*: *Arabidopsis thaliana* cysteine proteinase inhibitor; *Avd*: avidin; *Bt*: *Bacillus thuringiensis* δ-endotoxin gene; *CII*: serine proteinase inhibitor from soybean; *CpTi:* cowpea trypsin inhibitor; *cryIA*, *cryIA(a)*, *cryIA(c)*, *cryIAc646*, *cryIB*, *cryIIIA*, *cryIIIA(c)*: *Bacillus thuringiensis* δ-endotoxin genes; *gna*: snowdrop lectin gene; *KTi3:* Kunitz proteinase inhibitor; *OCI*: cysteine proteinase inhibitor from rice; *PI IV*: proteinase inhibitor from soybean; *pinII*: potato proteinase inhibitor II; *POD:* tobacco anionic peroxidase; *PtdPPO*: polyphenol oxidase gene from hybrid poplar; *Sbcys*: soybean cysteine proteinase inhibitor; *Stv*: streptavidin; *TDC1*: tryptophan decarboxilase gene

The role of insect pest resistance genes, such as those coding for the δ-endotoxins from *Bacillus thuringiensis* (*Bt*) and different proteinase inhibitors (PIs), has been investigated in several plant perennial species. Alternative strategies based on new insecticidal products and information about IPM practices are also reported. The direct insertion of these agronomically valuable traits into forest and fruit trees by genetic engineering is expected to integrate and accelerate the extensive classical breeding programmes already started for commercially important genotypes. Finally, it is worth noting that the production of genetically modified forest and fruit trees expressing multiple transgenes is considered an innovative research front, able to provide multitoxin resistance against different insect pests (Halpin 2005). Protocols for combining transgenes involve cross-fertilization, retransformation strategies and testing systems for selectable marker gene removal. Based on the promising results obtained with annual plant species, transgene stacking in forest and fruit trees can be considered a feasible strategy.

12.2 The Insecticidal δ-Endotoxins from *Bacillus thuringiensis* and their Role in the Control of Insect Pests

The soil bacterium *B. thuringiensis* produces a wide range of proteins (δ-endotoxins) that are included in crystals formed during sporulation and characterised by distinct insecticidal spectra (de Maagd et al. 2001). *Bt* spores contain high levels of δ-endotoxins harmful to specific insects that belong to the Lepidopteran, Dipteran and Coleopteran orders and are known as the major pests of annual crops and perennial tree species. The *Bt* spores and the crystal (Cry) proteins are ingested by the insect and solubilized within the alkaline midgut. The protoxins are then activated by proteinases and finally the active *Bt* toxin binds to specific molecular receptors causing the irreversible damage of the midgut epithelium by colloid osmotic lysis (Knowles and Dow 1993). *B. thuringiensis* has been used as a commercial insecticide for more than 50 years and to date an extensive number of reports have demonstrated that *Bt* proteins have negligible potential adverse effects against humans, animals and non-target invertebrates (Shelton et al. 2002). More than 130 *Bt* genes encoding different δ-endotoxins have been isolated and, among this extremely large gene array, those coding for the CryIA(a) and CryIA(c) proteins have been used to develop transgenic trees resistant to Lepidoptera (Table 12.1). In addition, the CryIIIA(a) protein has been chosen by different research groups to specifically target Coleopteran pests. The first generation of *Bt* trees expressing wild-type genes was characterised by extremely low levels of insect pest resistance due to the inefficiency of the bacterial codon usage in plants. The ability to withstand dangerous insects was subsequently increased using synthetic *Bt* genes containing coding sequences

that were adapted to the plant transcriptional and translational machinery. It is worth noting that all the different *Bt* genes used in these studies were placed under the control of the enhanced 35S Cauliflower Mosaic Virus (CaMV) promoter.

12.2.1 Transfer of *Bt* Genes into Forest Tree Species

The genus *Populus* is considered a model system in forest tree biotechnology due to the availability of effective transformation and regeneration protocols developed for an increasing number of species and hybrids and to other favorable features (Taylor 2002; Confalonieri et al. 2003). The first *Bt* poplar was obtained in 1991 by McCown and coworkers who used particle bombardment to introduce a partially modified *CryIA(a)* gene in a *Populus alba* × *Populus grandidentata* genotype. One of the regenerated *Bt* lines was able to significantly affect in greenhouse the growth of two main pests, the forest tent caterpillar *Malacosoma disstria* Hübner (Lepidoptera, Lasiocampidae) and the gypsy moth *Lymantria dispar* L. (Lepidoptera, Lymantriidae), which are responsible for severe damage to poplars (Robison et al. 1994). The same transgenic line, subsequently evaluated during the field-growing season, maintained high expression levels of the CryIA(a) δ-endotoxin after winter dormancy (Kleiner et al. 1995). The wild-type *CryIAc646* sequence was transferred to *Populus nigra*, *P. alba* and *Populus tremula* × *P. alba*. However, insect bioassays performed on transgenic *P. nigra* lines with larvae of *Hyphantria cunea* Drury (Lepidoptera, Arctiidae) and *L. dispar* did not show significant differences in larval mortality (Balestrazzi et al. 1994). When the bacterial *CryIIIA* gene was expressed in a *P. tremula* × *Populus tremuloides* genotype, the resulting GM plants were able to induce mortality of the leaf beetle larvae *Chrysomela tremulae* Fabricius (Coleoptera, Chrysomelidae) although transgene expression was detected only by reverse transcriptase-polymerase chain reaction (RT-PCR) (Cornu et al. 1996). A partially modified *CryIB* gene, encoding a protein toxic to the cottonwood leaf beetle *Chrysomela scripta* Fabricius (Coleoptera, Chrysomelidae) and forest tent caterpillar, was transferred to the *P. nigra* × *Populus maximowiczii* hybrid clone 'NM6' and feeding assays revealed a decrease of larval feeding in one of the tested transgenic lines (Francis 1996).

Poplar plantations in China are constantly threatened by defoliators such as the poplar lopper *Apocheima cinerarius* Erschoff (Lepidoptera, Geometridae) and the gypsy moth. Insect-resistant transgenic *P. nigra* plants were obtained by *Agrobacterium*-mediated genetic transformation using the *CryIA(c)* gene (Tian et al. 1993; Wang et al. 1996) and field evaluation was subsequently carried out on fourteen transgenic *P. nigra* lines in Manas (China). Results from this research showed that *Bt* poplars were protected against the damage caused by the larvae of the two main defoliators *A. cinerarius* and *Orthosia incerta* Hufnagel (Lepidoptera, Noctuidae)

(Hu et al. 2001). Interestingly, cross-protection of non-transgenic trees located in the same plantation was also observed. Several transgenic lines of *Populus trichocarpa* × *Populus deltoides* and *Populus* × *euramericana* hybrids carrying a *CryIIIA* gene were produced by Meilan et al. (2000). Transgenic plants showed very low feeding damage when infested by *C. scripta* larvae under natural conditions. More recently, Génissel et al. (2003) reported the expression of a synthetic *CryIIIA(a)* gene, specifically targeted to Coleoptera, in the hybrid poplar (*P. tremula* × *P. tremuloides*) clone INRA 353-38. In this construct the *Bt* gene was controlled by a hybrid promoter containing elements from both the 35SCaMV and the nopaline synthase promoters. The *Bt* toxin, found in mature leaves at a level corresponding to 0.05–0.0025% of the total soluble proteins, was lethal to *C. tremulae* at all developmental stages.

In contrast to poplar, *Eucalyptus* species are recalcitrant to in vitro propagation and genetic transformation. For these reasons, a limited number of transgenic studies is currently available (Campbell et al. 2003). *Eucalyptus* trees represent a valuable source of hardwood timber and pulp for paper. Plantations in Australia can be rapidly defoliated by insect pests such as the Tasmanian eucalypt leaf beetle *Chrysophtharta bimaculata* Olivier (Coleoptera, Chrysomelidae). The *CryIIIA* gene was introduced into *Eucalyptus camaldulensis* under the control of the pea plastocyanin gene promoter in order to direct accumulation of the protein to the young expanding leaves, tissues usually attacked by insect pests (Harcourt et al. 2000). One of the regenerated transgenic lines was able to confer resistance to early instars of *C. bimaculata* and *Chrysophtharta agricola* (Chapuis) and to the native chrysomelid beetle *Chrysophtharta variicollis* (Chapuis). This was the first useful trait introduced into a commercially relevant eucalypt species.

An emerging field in forest tree biotechnology is certainly represented by the genetic transformation of conifers with genes improving productivity (Tang and Newton 2003). The use of particle bombardment to transform conifers and the regeneration of genetically modified trees expressing genes for insect pest resistance was first reported by Ellis et al. (1993). In this study, the transgenic *Bt* white spruce (*Picea glauca*) plants were resistant to the spruce budworm *Choristoneura fumiferana* Clemens (Lepidoptera, Tortricidae). A *Bt* gene was subsequently transferred into the European larch (*Larix decidua*) by *Agrobacterium*-mediated genetic transformation and the regenerated plants were able to withstand attacks from the larch casebearer *Coleophora laricella* Hübner (Lepidoptera, Coleophoridae) (Shin et al. 1994). More recently, a synthetic *CryIA(c)* gene was introduced into the loblolly pine (*Pinus taeda* L.) by direct gene transfer (biolistics) to mature zygotic embryos (Tang and Tian 2003). Three different genotypes (J-29, E-11 and E-44, respectively) were used in this study. Feeding bioassays demonstrated that the transgenic plants were resistant to *Dendrolimus punctatus* Walker (Lepidoptera, Lasiocampidae) and *Cryptothelea formosicola* Strand (Lepidoptera, Psychidae), considered among the major pests threatening this forest species. A positive correlation between the presence of the δ-endotoxin

in needle extracts from the *Bt* plants and insect pest resistance was also observed. Transgenic plants of *Pinus radiata* expressing the *CryIA(c)* gene were obtained by biolistic transformation of embryogenic tissue (Grace et al. 2005). Bioassays carried out using larvae of the painted apple moth (*Teia anartoides* Walker) revealed variable levels of resistance.

The major role played by poplars in the research carried out to engineer insect pest resistance into forest trees is clearly evidenced in Table 12.1.

12.2.2 Transgenic Fruit Trees Expressing *Bt* Genes

Genetic engineering of the woody fruit plants has been hampered by the necessity to optimize transformation/regeneration systems suitable for the most recalcitrant species. Notwithstanding these difficulties, improved transgenic fruit species expressing agronomically relevant traits have been described as in the case of the major fruit tree crop *Citrus* (Gomez-Lim and Litz 2004). Unfortunately, only a few reports are currently available on insect pest resistance as shown in Table 12.1 where only apple and walnut trees are listed. James et al. (1993) reported the production of transgenic apple (*Malus* × *domestica*) plants with a *CryIA(c)* gene; however, results from feeding assays are not available. The codling moth *Laspeyresia pomonella* L., known as a major insect pest of apple and pear, causes severe damage to walnut (*Juglans regia*) production. The wild type *CryIA(c)* gene inserted in walnut somatic embryos resulted in inadeguate expression levels (Dandekar et al. 1994), while the use of a synthetic *CryIA(c)* gene allowed expression levels adequate to control *L. pomonella* (Dandekar et al. 1998). Sixty-one GM embryo lines were obtained and tested with first instar codling moth larvae. In 34% of these lines, 80–100% mortality was observed in the presence of detectable amounts of the δ-endotoxin. The *Bt* protein was toxic to codling moth larvae at a very low concentration, corresponding to 0.01% of the total cellular proteins.

The increasing availability of protocols for gene transfer widely applicable to different genotypes and species will hasten the production of new varieties of insect-resistant fruit trees.

12.3 Plant Proteinase Inhibitors: A Useful Tool for Plant Defence Against Insect Predation

Proteinase inhibitors (PIs) are natural compounds abundantly found in seeds and storage organs of a wide range of plant species and contributing to the plant defence system against insect pest and pathogens (Schuler et al. 1998). Proteinase inhibitor families which are specific for each of the four classes of proteolytic enzymes (cysteine, serine, aspartic and metallo proteinases) have

been identified (Ryan 1990; Heitz et al. 1999). The inhibiting activity of PIs is due to the ability to form stable complexes with proteinases, blocking, altering or preventing the access to the substrate-binding region of their catalytic site (Haq et al. 2004). In order to develop effective strategies for plant protection against insect pests based on PIs transgenesis, it is imperative to know the class of proteolytic enzymes present in the insect guts. Different proteinases predominate in different insects: many Lepidopteran species have serine proteinases as the major digestive enzymes, whereas Coleoptera have a wider range of dominant gut proteinases (Koiwa et al. 1997; Schuler et al. 1998). The antimetabolic action of PIs in the digestive system of insects is not completely understood: Broadway and Duffey (1986) suggest that the PI-mediated inhibition of proteinases is responsible for hyperproduction of digestive enzymes, enhanced loss of essential aminoacids and finally inhibition of insect growth rates. However, some insect species seem to be able to modify dynamically the spectra of their digestive enzymes by the production of insensitive proteinases (Jongsma et al. 1996; Ferry et al. 2004). Because of this ability, the use of PI-coding genes as source of resistance to insect pests for tree improvement programs not always represents a winning strategy. At least eight different genes encoding plant PIs have been transferred into forest and fruit trees as reported in Table 12.1, and most of them belong to the Fabaceae and Solanaceae families where they are targeted mainly against Lepidoptera but also against some Coleopteran insects.

12.3.1 Transfer of *PI* Genes into Forest and Fruit Trees

To date, the use of PIs transgene technology has been reported mostly for different poplar species and their hybrids (Table 12.1). Additional studies concerning the expression of *PI* genes in other woody plants and fruit trees are probably covered by confidential agreements or patent applications. The first report of PIs-engineered trees was presented by Klopfenstein et al. (1993) who transformed two different hybrid poplars (*P.* × *euramericana* and *P. alba* × *P. grandidentata*) with the potato serine proteinase inhibitor *pin2* gene. Feeding experiments with cottonwood leaf beetle (*C. scripta*) larvae revealed a decrease in the consumed leaf area and in the average weight of larvae fed on transgenic *P.* × *euramericana* leaves when compared to controls (Kang et al. 1997). A feeding bioassay performed with the willow leaf beetle *Plagiodera versicolora* Laicharting (Coleoptera, Chrysomelidae), revealed that one out of eleven transgenic *P. alba* × *P. grandidentata* lines exhibited significantly less consumed tissue than the control plants (Heuchelin et al. 1997). Pappinen et al. (1995) engineered silver birch (*Betula pendula*) with the same *pin2* gene. However, the transgenic birch did not show higher trypsin inhibitory effect when compared to the controls. The transfer and stable integration of the *CpTi* gene, encoding a trypsin inhibitor from cowpea, into apple (*Malus* × *domestica*, cv. Greensleeves) was reported by James et al.

(1993) but information concerning the level of insect pest resistance in these genetically modified plants are missing. Transgenic black poplars (*P. nigra*) overexpressing the *KTi*$_3$ gene encoding a Kunitz serine proteinase inhibitor have been produced (Confalonieri et al. 1998). Although the trypsin-like digestive proteinases of the polyphagous moth *L. dispar* and *Clostera anastomosis* L. (Lepidoptera, Notodontidae) were inhibited in vitro by the KTi$_3$ protein from transgenic plants, feeding assays performed with larvae of the same insect pests showed that KTi$_3$ did not cause significant larval mortality and inhibition of growth, as well as pupal weight. The same authors obtained transgenic *P. nigra* plants expressing the *CII* and *PI-IV* serine proteinase inhibitor genes, but the evaluation of these plants for insect pest resistance has not been reported (Confalonieri et al. 1997).

Genes coding for different cysteine proteinase inhibitors from rice, soybean and *Arabidopsis thaliana* have been introduced into poplars. In the first case, Leplé et al. (1995) reported the production of transgenic *P. tremula* × *P. tremuloides* plants expressing an oryzacystatin (*OCI*)-encoding gene. Transformants expressing OCI at a level up to 2% of total proteins were analyzed by in vitro feeding bioassays and showed significant levels of resistance to the poplar leaf beetle *C. tremulae*. A field trial of selected *OCI* transformants and control plants was started with the aim of testing the stability of transgene expression under natural conditions (Leplé et al. 1999). A 450-bp synthetic sequence, encoding a soybean cystatin proteinase inhibitor, was inserted into white poplar (*P. alba*) (Delledonne et al. 1998). However, these transgenic lines were not further evaluated, due to the very low expression level detected by northern analysis. More recently, the same authors engineered a white poplar cultivar with a novel *A. thaliana* cysteine proteinase inhibitor (*Atcys*) gene (Delledonne et al. 2001). The level of papain inhibitory activity in *Atcys* transformants was sufficient to inhibit most of the digestive proteolytic enzymes of the chrysomelid beetle *Chrysomela populi* L. (Coleoptera, Chrysomelidae). Feeding bioassays demonstrated that, among the three tested genetically modified lines, two showed total resistance to *C. populi* larvae. The same *Atcys* gene was used to engineer an apple rootstock (*Prunus malus*, cv. M9) against the cockchafer *Melolontha melolontha* L. (Coleoptera, Scarabaeidae). Accumulation of *Atcys* transcript was detected in several transgenic plant lines by RT-PCR analysis and feeding bioassays are currently in progress (Romanò et al. 2004).

Despite these promising results, no forest and fruit trees expressing *PI* genes have been commercialized. Several genetic and/or environmental factors can influence the level of PIs transgene expression, while spatial and developmental regulation might be required. Furthermore, the capacity of target insects to adapt to PIs and the possible evolution of insect biotypes not susceptible to specific inhibitors seems to confirm the difficulties in constructing an effective plant defence system against insect pests. A deep knowledge of the specific activities and interactions of proteinase inhibitors, their physiological effects and the response of insects to PIs exposure is

required. In order to improve the effectiveness of PIs transgenesis against insect pests of fruit and forest trees, the use of large-spectrum inhibitors against all the digestive proteinases of insect pests and the isolation of new useful proteinase inhibitors from unrelated organisms or by in vitro mutagenesis may represent the future goals in this research field.

12.4 Other Strategies to Obtain Insect Resistance in Forest and Fruit Trees

In recent years, two major groups of genes have been used to control insect predation on woody perennial species: those encoding proteinase inhibitors of digestive enzymes and those coding for the *Bacillus thuringiensis* δ-endotoxins (Confalonieri et al. 2003). However, both these strategies did not always result in very high levels of pest control while the possible occurrence of insect resistance represents a limit to the use of transgenic trees. For these reasons a constant search for novel sources of resistance has been carried out and different strategies have been proposed to engineer trees for resistance to insect pests (Table 12.1). The immature and mature leaves of transgenic sweetgum (*Liquidambar styraciflua*) expressing tobacco anionic peroxidase, an enzyme generally associated with resistance to plant pathogens, showed increased resistance to *H. cunea* and *L. dispar* (Dowd et al. 1998). Expression of an insect-selective neurotoxic polypeptide (AaIT) derived from scorpion venom (*Androctonus australis* Ewing) in transgenic hybrid poplar (*P. deltoides* × *P. simonii*) decreased leaf consumption and larval weight of *L. dispar* and resulted in a higher larval mortality rate (Wu et al. 2000). A different approach was used by Markwick et al. (2003) who engineered an apple (*Malus* × *domestica*) genotype with genes encoding the biotin-binding proteins avidin and streptavidin. These products, responsible for vitamin deficiency in insects, are known to be harmful to a range of different insect species (Markwick et al. 2001). Feeding experiments with larvae of the lightbrown apple moth *Epiphyas postvittana* Walker (Lepidoptera, Tortricidae) revealed the presence of high insect resistance level in the transgenic apple plants expressing both avidin and streptavidin. A promising approach for increasing insect pest resistance relies on the expression of genes encoding enzymatic activities that allow plants to accumulate new metabolites or to increase the level of endogenous compounds involved in protection mechanisms.

Recently, an hybrid poplar (*P. tremula* × *P. alba*) was engineered with the *TDC1* gene encoding tryptophan decarboxylase from *Camptotheca acuminata* (Gill et al. 2003). This enzyme catalyzes the conversion of tryptophan to tryptamine. The transgenic poplar plants showed elevated levels of tryptamine that were associated with the observed reduction in the growth rate of forest tent caterpillar (*M. disstria*) larvae. Another interesting research field,

based on the use of polyphenol oxidase (PPO), allowed the indirect modification of insect resistance in transgenic forest trees. Wang and Constabel (2004) transformed *P. tremula* × *P. alba* with the *PtdPPO* gene from *P. trichocarpa* × *P. deltoides*. Polyphenol oxidase overexpression in transgenic poplar provided, under certain conditions, significant levels of resistance against *M. disstria* larvae. Recovery of transformed perennial tropical fruits has been referenced by Yang et al. (2000) which engineered grapefruit (*Citrus paradisi*) with a *Galanthus nivalis* (*gna*) insecticidal lectin gene. However, the evaluation of transgenic plants against *Citrus* insect pests was not reported.

Although a lot of work has been carried out using transgene technology, the effectiveness of insect-resistant transgenic trees should be demonstrated under long-term field conditions.

12.5 Environmental Risk and Deployment Strategies for Genetically Engineered Insect-resistant Trees

Field-growth and performance of GM trees need to be evaluated over several years in order to assess their potential impact on human health and environment and obtain information relevant to commercial use. Small-scale trials are not recommended due to the long life cycle of perennial plants. Field trials of transgenic poplars expressing *Bt* and proteinase inhibitor genes are currently performed, as described in the following paragraph. The environmental safety of GM trees is considered a primary issue for their commercial use. For this reason, the potential risks associated with their release should be compared to the actual benefits and carefully assessed according to a concept of "substantial equivalence" with the non-GM strategies (Shelton 2004). Some risks are of general concern (gene flow to wild populations, invasiveness of GM trees, food safety issues, spread of marker genes responsible for antibiotic resistance) whilst some others specifically refer to the insect-resistant engineered trees and will be discussed below. In addition, the use of multiple strategies (IPM, Integrated Pest Management) to ensure the control of invasive pests is reported. Such strategies need to be utilized and combined according to specific rules, derived from multidisciplinary experiences, in order to fulfill expectations.

12.5.1 Field Trials with Insect-resistant GM Trees

Accurate estimation of the total number of field trials currently performed with insect-resistant forest and fruit trees is considered a difficult task since not all the activities can be easily reached using both public databases and regulatory sites (FAO 2004). As reported by James (1997), field tests were started for at least seven tree species engineered for insect resistance. Kleiner

et al. (1995) evaluated the field performance of *P. alba* × *P. grandidentata* plants engineered with the *CryIA(a)* gene. The GM poplars were planted in the summer of 1993 and insect resistance was tested after winter dormancy (June, July and August 1994, respectively). Bioassays carried out with larvae of the forest tent caterpillar and gypsy moth revealed that the first one was more susceptible to the CryIA(a) toxin. A long-term field trial was also established with the poplar hybrid *P. tremula* × *P. tremuloides* engineered with a cDNA coding for an oryzacystatin (OCI) proteinase inhibitor (Leplé et al. 1999).

According to a recent study, 21 field trials refer to GM insect-resistant forest trees. The United States are involved in the majority of them, followed by China which has rapidly expanded its research activity on GM trees production and applications (FAO 2004). Transgenic hybrid poplars (*P. trichocarpa* × *P. deltoides* and *P.* × *euramericana*) expressing the *CryIIIA* gene have been tested under natural conditions and showed very low feeding damage by *C. scripta* larvae (Meilan et al. 2000). Field evaluation of GM *P. nigra* trees expressing the *CryIA(c)* gene was performed during 1994–1997 in Manas (China) and data collected showed that the leaf damage, the insect larval density and the number of pupae per unit area in the soil were significantly lower, thus excluding the need for chemical protection (Hu et al. 2001). Moreover, the insecticidal activity supplied by the GM poplars was responsible for cross protection toward the non-transgenic trees cultivated in the same plantation. In China, field trials expanding up to 80 ha on eight sites in seven different provinces have been subsequently established since 1999, using the same engineered *P. nigra* trees (FAO 2004). Field trials were also carried out using the transgenic hybrid poplar clone 741 expressing simultaneously the *CryIA(c)* and the arrowhead proteinase inhibitor (*API)* genes (Zheng et al. 2000). A three-year old plantation established with the GM 741 poplars and monitored in terms of species composition, dominance and community structure of the defoliating insects and their natural enemies (Gao et al. 2003).

12.5.2 Toxicity and Allergenicity of Proteins Encoded by Genes for Insect Pest Resistance

Fruit trees such as walnut (*Juglans spp.*) and apple (*Malus spp.*) have been transformed to express *Bt* proteins aimed at the control of Lepidopteran pests in the United States (Krattiger 1996). The food safety of *Bt* proteins has been exhaustively reviewed by Shelton et al. (2002) who reported the results of several studies mainly carried out by the U.S. Environmental Protection Agency (US EPA). Feeding assays of all the tested *Bt* proteins did not show any significant effect when mice were fed with a pure preparation at doses >5000 mg/kg bodyweight (US EPA 2000). Long-term studies were not carried out as the instability of the protein in digestive fluids eliminates this need (US EPA 2000). Potential allergenicity was assessed by in vitro digestion assays and

examining the level of amino acid homology against a database of known allergens (WHO 2000). Based on these tests, it was concluded that "it is unlikely that consumers would develop allergic sensitivity after oral exposure to transgenic foods that currently contain the gene encoding the *Bt* protein" (Felsot 2000). In contrast to concerns about toxicity and allergens from the genetically modified organisms (GMOs), there is evidence for health benefits from GM crops such as *Bt* corn (APS 2001).

12.5.3 Development of Target Pests Resistant to GM Trees

Adaptations by insects to altered sources of food, plant quality, quantity and distribution are well documented in natural and managed ecosystems (Singer and Parmesan 1993). As a consequence, the development of pest resistance to GM trees is likely to occur, with an evolution rate depending on the genetic basis of resistance, the initial frequency of resistance alleles in the population, the competitiveness of resistant individuals in the field, and the resistance management strategy adopted (Shelton et al. 2002). Moreover, in the particular case of GM trees the enormous differences between pest and host generation times can greatly reduce the efficacy of any delaying tactics (Raffa et al. 1997). The poplar leaf beetle *C. tremulae* rapidly evolved resistance to sprays of *Bt* toxins in laboratory and in field (Augustin et al. 2004), thus confirming that resistance management for *Bt* plants remains a serious concern.

The high dose/refuge strategy requires the use of non-transgenic insect-susceptible cultivars that are cultivated in the field (typically 20% of the total plantation). The refuge has the fundamental function to maintain the insect population carrying alleles responsible for susceptibility. In the field, susceptible insects will mate with the rare resistant insects that survive on the transgenic plants. The heterozygous susceptible progeny is killed by the insecticidal protein synthesized by GM plants (Gould 2000). In order for this strategy to prove effective, both the frequency of alleles conferring resistance and the survival of heterozygous resistant individuals must be low (Andow and Hutchison 1998). At the same time, the occurrence of random mating between resistant and susceptible genotypes is required (Génissel et al. 2000). As regards *Bt* poplars, the resistance in *C. tremulae* was found to be completely recessive (Augustin et al. 2004). In *Bt* cotton, the optimal spatial configuration of refuges has been modelled in order to improve effectiveness and reduce costs; in particular, aggregated distributions of nontransgenic plants and production of lower toxin doses in *Bt* plants are suggested (Vacher et al. 2003). Other models showed that 'active' refuges are more effective in preventing the evolution of resistance in insects: in this case refuges are made by different GM plants producing a toxin which actively selects against the resistant genotypes evolved on the insect-resistant crop (Pittendrigh et al. 2004). Although planting refugia for susceptible insects represented the main current management strategies for transgenic crops aimed at reducing biotype

evolution, further studies are needed to better define the optimal refugia in term of size and spatial distribution, depending on the rates and distances of insect dispersal (James 1997). In the case of transgenic trees, the application of such strategies might be not easily amenable due to the long-lived nature of these species combined with the constant production of toxins in most plant tissues. Other mitigation strategies aimed at reducing the selective pressure on pests have been suggested, based on the use of inducible promoters or by targeting the transgene expression to a specific developmental stage of the plant organ: under these conditions, predation could be tolerated before the plant transgenic defenses are activated (Ellis et al. 1996). Diversifying tactics seem to provide good chances in delaying evolution of resistant pests: plantation of mosaics comprising both engineered and non-engineered trees, introduction of multiple resistance genes showing highly different modes of action, synergists to increase toxicity, rotation of toxins, use of biological control agents and pheromones, integration with other silvicultural practices (Raffa 1989), use of transgene-based resistance as a supplement to existing quantitative resistance developed through conventional breeding (Burdon 1999) may represent effective strategies. As a matter of fact, despite resistance to *Bt* crops most likely remains a question of not "if" but "when", and eight years later the first commercialization of *Bt* cotton and corn (which have been grown on a cumulative area greater than 80 million ha worldwide), resistance has yet to be documented (Bates et al. 2005). This means that *Bt* crops have already exceeded the length of time typically required in the field before resistance to most conventional neurotoxic pesticides is first documented (McCaffrey 1998).

12.5.4 Emergence of New Pests Following GM Trees Deployment

This event is quoted to have occurred in China in *Bt* poplar plantations (Rautner 2001). Problems associated with new secondary pests are known also for *Bt* cotton (Bachelor 2000). The inconvenient could be enhanced by longevity of trees compared to annual crops, but interpreting such results is complicated, due to the complexity of the insect communities hosted on trees. Moreover, most studies can be conducted only on a small scale and during a relatively short period of time, thus providing inadequate information (Shelton et al. 2002).

12.6 Deleterious Effects on the Ecosystems

In general, GM trees should be less directly damaging to natural enemies of pests than are traditional pesticides (Raffa et al. 1997). However it seems inevitable that any human intervention to protect crops, reducing the pest

population, will entail some disruption of tritrophic systems. Resistant transgenic plants are likely to lead to a reduction in host quality and so may affect larval parasitoids indirectly: this can occur both with *Bt* plants and with plants expressing enzyme inhibitors and lectins, which are generally less resistant to pests but affect a wider range of species (Schuler et al. 1999). Two reports showing adverse effects on lacewings feeding on larvae that had ingested *Bt* corn (Hilbeck et al. 1998) and on the monarch butterfly through pollen consumption (Losey et al. 1999) received considerable attention, but both reports were criticized for their inappropriate design, methodology and interpretation (Hodgson 1999; US EPA 2000). These studies demonstrate the difficulty in performing laboratory assays that have relevance in the field, as interactions in laboratory, although dramatic, may not be realistic in the field (Shelton et al. 2002). Laboratory test species should be selected, considering their ecological role in the agro-ecosystems and tests should be carried out under ecologically realistic laboratory 'worst case' scenarios (Lövei and Arpaia 2005). Also, the risk assessment on the potential adverse effects on soil organisms of GM crops is especially difficult because our basic understanding of nutrient cycling, detritivore taxonomy and ecology, and soil biochemical and biophysical processes is limited (Raffa et al. 1997). The effects depend on the gene product and the soil properties: *Bt* toxins and proteinase inhibitors active against Coleoptera pose some concern, as beetles are important components of the soil fauna. The consequences deriving from the presence of a stable gene product must also be considered, as buildup in the soil over long periods may represent a problem (Raffa et al. 1997). A comparison between soil organisms found in fields cultivated with *Bt* and non-*Bt* crops and between soils amended with *Bt* or non-*Bt* biomass showed no significant differences (Saxena and Stotzsky 2001).

12.7 Horizontal Transfer of the Transgenes to Other Organisms

Both virus and bacteria possess the potential to incorporate transgenes into their genomes, leading to the expression of novel and possibly undesirable traits (Pretty 2001). Following field sprays, horizontal transfer of the *Bt* genes to *Bacillus mycoides* has been demonstrated in the soil, where *Bt* and its toxins can persist for long periods (at least 88 and 28 months, respectively) after spraying (Vettori et al. 2003), giving some concern on the fate in the soil of the *Bt* genes present in GM plant residues. Currently, the available literature strongly suggests that long-term investigation is needed, especially when long-living Organisms such as trees are concerned.

12.8 Conclusions

The global production system for long-lived tree species continuously deals with problems originating from pest attacks and available control tactics: exposure of insects to chemical pesticides promotes the evolution of resistant populations, eliminates beneficial predators, parasites and other non-target organisms and often results in secondary pest outbreaks. Moreover, invasion of both indigenous and introduced pests related to unexpected climatic changes may occur. Appropriate multiple-control strategies are required in order to minimize hazards to humans and environment and allow a stable increase in agricultural production. Within this context and in view of recent progress, research in basic biology and molecular biotechnology needs to be strongly supported. Among the biotechnological strategies, the use of genetic engineering for tree improvement offers a new and powerful tool to enhance significantly wood and food yields: the gene pool of commercially important genotypes can be expanded by adding novel genes conferring insect pest resistance. In order to investigate the level of transgene stability in response to variable environmental conditions and to assess the potential ecological risks deriving from GM plantations, extensive field trials with transgenic insect-resistant trees are required. A regulatory approach ethically bound to preserve resource sustainability, ecosystem integrity and environment preservation is urgently needed (James 1997). Moreover, regulatory requirements will be essential to determine the safety of GM products from long-lived tree species. When these issues will be addressed, more benefits to productivity and quality of forest and fruit trees could be provided.

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13 Towards Genetic Engineering for Drought Tolerance in Trees

Andrea Polle[1], Arie Altman[2], and Xiangning Jiang[3]

13.1 Introduction

Drought has a major impact on plant production and productivity, since water is required in all plant physiological processes. Water is the major medium for transport of nutrients and metabolites and is the solvent, in which all biochemical reactions of the living cell take place. At the physiological level, the water status of a plant is expressed by its water potential or as the relative water content. Under normal conditions, when water availability is sufficient, essentially all water entering land plants comes from the soil through the roots. The water is then conducted to other parts of the plant, first to the xylem, where long-distance transport takes place, and then to the leaves, where the water is transpired into the surrounding atmosphere. The amount of water released into the atmosphere is regulated by a plant's stomata. As a rule of thumb, during a growing season about 1000 times more water is transpired by a plant than remains in it for growth (Nobel 1994).

13.2 Water as a Central Molecule in Plant Physiology

The flux of water in the soil-plant-atmosphere continuum is driven by the difference in water potential between soil, plant, and atmosphere. By definition, the water potential (Ψ is zero in pure water (1 atm, 25 °C) and decreases with decreasing water "availability", i.e. in solutions, in vapour, etc. This can be illustrated by a few examples: in well-watered soil the water potential is –0.3 MPa; inside plants it is about –0.6 MPa; within the stomatal cavity it can reach about –6.9 MPa (=95 % relative air humidity), and outside in the atmosphere it is –95.1 MPa (=50 % relative air humidity; Nobel 1994). The movement of water molecules follows the water potential gradient from the soil to the atmosphere. If soil water availability decreases, the plant needs to adjust, i.e. lower, its internal water potential to be able to continue extracting water from the soil. The

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water potential of a plant, usually measured in the xylem, is therefore a suitable measure for the extent of drought stress experienced by a plant (Scholander et al. 1964; Cochard et al. 2001). Within a tissue, water flux is determined by the concentration of solute molecules inside and outside the plant cell. The radial cell-to-cell transport across membranes is facilitated by aquaporins (Chrispeels and Maurel 1994; Kirch et al. 2000; Tyerman et al. 2002). Some aquaporins can also transport small solutes such as glycerol, other small organic molecules, and ions (Quigley et al. 2002; Tyerman et al. 2002), which may be relevant for osmotic adjustment. The extent to which aquaporins contribute to plant water status under favourable growth conditions and abiotic stress is not clear. Among various aquaporin genes, some were up regulated under stress (Yamaguchi et al. 1992; Yamada et al. 1997), whereas others such as PIP1a were not affected (Grote et al. 1998). PIP1b overexpression significantly increased plant growth rate, transpiration rate, stomatal density, and photosynthetic efficiency under well-watered conditions; but during drought stress, it had a negative effect, causing faster wilting (Aharon et al. 2003). Furthermore, the relative contributions of the apoplastic and symplastic routes to water transport in plants under favourable growth conditions, as well as under drought stress remain a matter of debate (Carvajal et al. 1998; Schaffner 1998; Amodeo et al. 1999; Tyerman et al. 1999). Changes in water availability may cause perturbations in solute flux and of cellular structures, alter the composition of the cytoplasm, and affect cellular functions.

Water deficits pose problems both at the cellular level, due to dehydration and turgor loss, and also for long-distance transport of nutrients. All aspects of water availability and transport are particularly relevant for growth and survival of tree species since trees have to ensure water transport into their crowns to heights of up 100 m. Additionally, trees have to acclimatise to changing water supply not only on a daily basis but also seasonally over many decades during their long life cycle.

13.3 Water as a Limiting Resource

The availability of water is a basic necessity for growth of plants. To date, soils too dry for crop production cover 28% of the Earth's land (http://www.un.org/earthwatch/desertification/). The problem of desertification is aggravating because of unsuitable ways of land utilisation occurring especially in developing countries. Natural forests are being continuously destroyed and are replaced by agriculture. If not suitable for this purpose any longer because of soil infertility, the land is abandoned and the area left to erosion and desertification. The growing population and the need for increasing agriculture have displaced forests to less fertile sites, which are not amenable for food production, such as shallow soils and steep slopes. These edaphic conditions are usually also accompanied by soil drought. Further problems are caused by changes in land use practices and by increasing water consumption, e.g. by damming or alterations in rivers

beds, which influence water availability by affecting the ground water table. This may lead regionally to destruction of natural ecosystems with serious problems for the inhabitants. For example, unsuitable water management in north-west China (Xinjiang Uygur Autonomous Region) is causing the decline of woody species important to protect sensitive desert ecosystems and oases from wandering dunes, thus, threatening the existence of these habitats (Wei 1991).

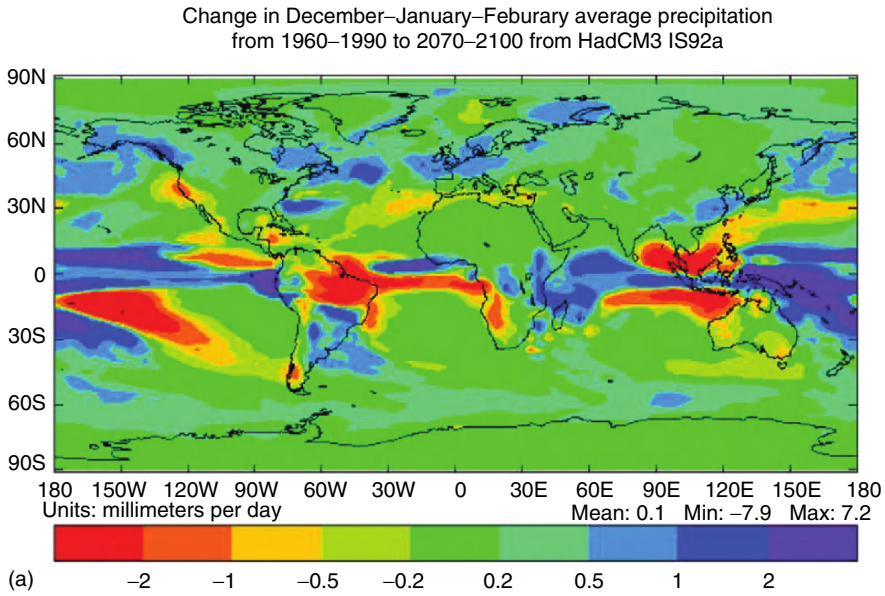
In addition to regional problems, forecasts of global climate change scenarios predict worldwide changes in precipitation patterns (Easterling et al. 2000), whereas in Central and South America, decreases in rain are predicted for the whole year (Fig. 13.1). In Europe, precipitation in winter months is expected to increase and to decrease in summer during the growth phase of the vegetation (Fig. 13.1).

From these considerations, it is obvious that we have an urgent need to understand drought tolerance mechanisms in forest trees, not only for improving stress tolerance of trees to combat acute problems but also to develop molecular markers to identify stress tolerant ecotypes, to preserve and exploit the natural variability for improved management of future forests. Towards the goal of engineering drought tolerant tree species, we must progress the procedures of genetic engineering of major forest tree species to understand how trees respond to drought, what are the differences and similarities with herbaceous plants, and which research strategies need to be implemented (Tzfira et al. 1998). Our present knowledge on drought responses in trees is mainly based on ecophysiological and biochemical studies (Chaves et al. 2003; Wang et al. 2003, 2004). In recent years, much progress has been made in elucidating signalling pathways involved in drought signalling at the cellular level in herbaceous model plants. These aspects are discussed in several excellent recent reviews (Seki et al. 2003; Bartels and Souer 2004; Vinocur and Altman 2005), and thus will be treated here mainly with respect to their significance for tree species. Transcriptional profiling and proteome analyses are novel techniques used to identify the molecular components leading to adaptation to water deficits. These techniques are promising tools, which will be discussed, especially with respect to their perspectives and limitations to increase our knowledge on drought tolerance mechanisms and how this information can be implemented to improve drought tolerance in tree species.

13.4 Signalling Cascades and Metabolic Stress Adaptation from the Cellular to the Organismic Perspective

13.4.1 ABA, MAPKK, Lipases, and Transcription Factors are Involved in Transmission of the Stress Signal

Perception of water deficits is complex and not yet fully understood. The identity of the primary sensor, which could be water status, bound water,



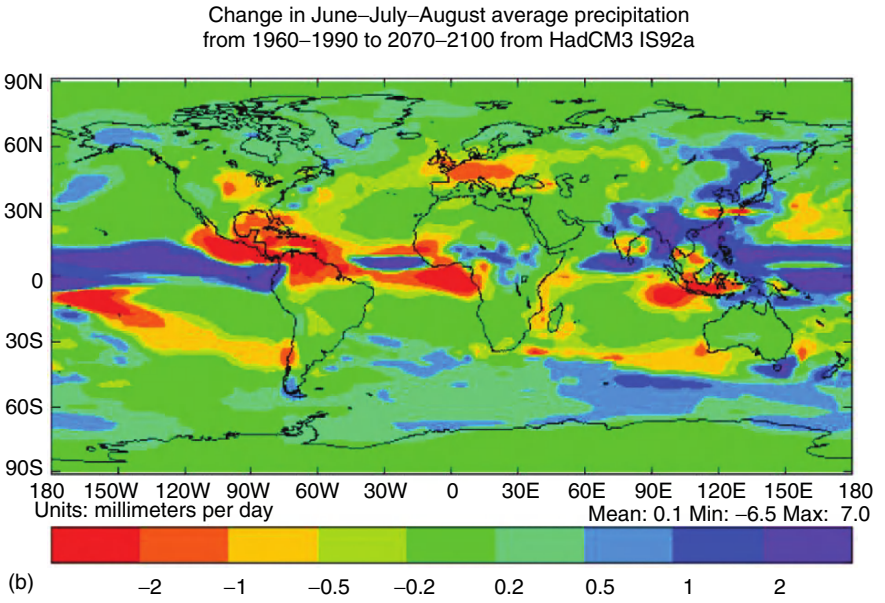


Fig. 13.1. Predicted changes in global precipitation patterns for the winter and the summer season. The period from 1960 to 1990 was taken as reference and changes are indicated in 1 mm/day on a scale from *red* to *blue*. Climate change predictions from the Hadley Centre: http://www.metoffice.com/research/ hadleycentre/models/modeldata.html

hormones such as abscisic acid (ABA), turgor, etc., is still questionable. Furthermore, signalling at the cellular level and long distance signalling have to be distinguished. In trees, long distance signalling is particularly important to transmit the message from the roots to the leaves. The nature of all these signals is also still unclear. Although ABA seems to be one of the components, others such as hydraulic pressure or electrical signals have also been considered (Comstock 2002).

The first step at the cellular level is the perception of the environmental signal. A transmembrane histidine kinase has been suggested to act as an osmosensor, triggering a down-stream signalling cascade, which leads to the activation of dehydration responsive genes (Urao et al. 1999). In Arabidopsis, osmotic stress activated the expression of AtHK1, the putative osmosensor (Urao et al. 1999) (Fig. 13.2). In *Eucalyptus spec*., two HKT1 homologues have been implicated in osmosensing (Liu et al. 2001). The subsequent signalling pathway involves several MAP kinases (mitogen activated like protein), which mediate protein phosphorylation and phosphatases, which catalyse dephosphorylation (Luan 1998; Lee et al. 1999) (Fig. 13.2). These events also lead to changes in cytoplasmatic Ca concentrations, which may integrate different stress pathways via the Ca-dependent protein kinases CDPK (Knight and Knight 2001).

The drought signalling pathway contains both ABA-responsive and ABA-independent elements (Seki et al. 2003; Bartels and Souer 2004) (Fig. 13.2). The physiological function of ABA in mediating stomatal closure, thereby diminishing water loss, has been the subject of many ecophysiological studies (Zeevaart and Creelman 1988) (see below). In addition to ABA, other genes whose drought-responsiveness is independent from ABA have been identified in ABA-deficient or insensitive mutants. For example, drought also activates phospholipid-signalling pathways. Phospholipase C, a member of one of these pathways, appears to be independent of ABA, whereas phospholipase D, which is involved in stomatal closure, is part of an ABA-responsive pathway (Bartels and Souer 2004). Genes of the ABA-independent pathway contain drought responsive elements motifs (DRE/CRT) in their promoters (Yamaguchi-Shinazaki and Shinozaki 2005). The DRE containing promoters seem to act as points of cross talk, where different abiotic stress signalling pathways converge.

Analysis of the signalling pathways in the model plant Arabidopsis has shown that different abiotic stress factors, such low temperature, drought, high light, etc. result in partially overlapping acclimatory responses. It has also been demonstrated in spruce that a provenance that was more resistant against freezing stress was also more resistant to drought (Blödner et al. 2005). This finding is probably of high practical importance for the selection of tree species for forest plantations since the trees currently planted are likely to encounter significant changes in their growth environment due to global change during their long life span.

ABA plays a most prominent role in mediating drought responses. It induces the expression of down-stream genes mediated through *cis*- and *trans*-acting transcription factors (Shinozaki and Yamaguchi-Shinozaki 2000;

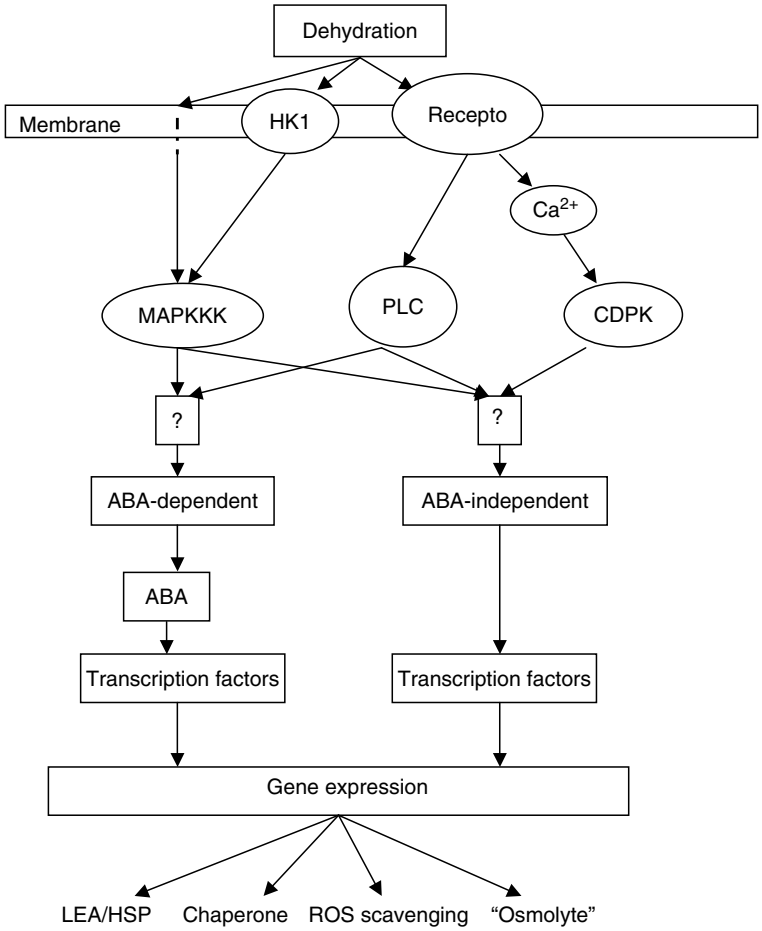


Fig. 13.2. Molecular responses to drought stress. Upon dehydration the cell activates an array of responses, starting with signalling cascades and leading to the activation of gene transcription and stress adaptation to acquire a new level of homeostasis (for details see text)

Zhang et al. 2005). Common to most of these genes is the presence of an ABA responsive element (ABRE) in their promotor (Uno et al. 2000). Several *cis*-acting transcription factors (BZip) have been cloned in Arabidopsis and were found to be up regulated under drought stress (Uno et al. 2000). These transcription factors activate in turn the transcription of drought-responsive proteins such as RD22 and RD29 (Yamaguchi-Shinozaki and Shinozaki 1993a,b; Abe et al. 2003). Notably, RD22 did not contain an ABRE motif but MYC and MYB binding domains (Abe et al. 1997). Overexpression of the MYB and MYC proteins in Arabidopsis resulted in increased ABA sensitivity and DNA-array

analysis of the overexpressors revealed up-regulation of many transcripts that were also inducible by ABA (Abe et al. 2003).

Little is known about drought signalling at the cellular level in trees. Although woody and herbaceous species may share similar principles of drought stress signalling, important differences with respect to the transmission or modulation of the pathways may exist, even at the cellular scale, compared with Arabidopsis. This assumption is supported by the observation that different species within one genus (*Populus*), as well as ecotypes of the same poplar species originating from different climatic conditions, differed in their stomatal responsiveness to ABA (Chen et al. 2002; Yin et al. 2004; Zhang et al. 2004a). Chen et al. (2002) suggested that polyamines and ethylene were involved as transducers modulating the intensity of the ABA signal. They found that drought resulted in increased ABA concentrations in the xylem sap of poplar trees, and in turn inhibited polyamine synthesis and increased ethylene concentrations. Polyamine concentrations declined in the drought-sensitive species and leaves were shed, whereas the tolerant species maintained higher polyamine levels and did not shed its leaves (Chen et al. 2002). When the polyamine, spermidine was fed via roots, drought-stressed conifer seedlings could sustain higher water loss before the turgor declined than stressed controls (Islam et al. 2003). These observations show that different plants do not respond in the same way to ABA because the signal may be tuned by interaction with other components, such as polyamines, which affect the degree of drought tolerance. However, to date we have only correlative evidence to support these ideas since experimental analyses of mutated or transgenic tree species are still lacking. It has also been suggested that polyamines may act as antioxidants (Guerrier et al. 2000) (see below). In transgenic rice overexpressing polyamine biosynthetic enzymes, a protective function of spermidine against drought injury was recently suggested (Capell et al. 2004).

Although ABA has, undoubtedly, an outstanding role in regulating stomatal aperture (Schroeder and Hedrich 1989), its function in long-distance root-to-shoot signalling in trees is questionable. In a study in which drought was experimentally induced in mature pine trees, stomatal conductance declined when the volumetric soil water content decreased below 0.12 (Perks et al. 2002). The decline in stomatal aperture was not mediated by ABA, since it preceded any increase in xylem ABA concentration. Using sap flow velocity measurements in 15 m tall pine trees, it was calculated that under normal conditions it would take a signal produced in roots about 12 days to reach its site of action in leaves (Perks et al. 2002). Under stress, which diminished sap flow, it would have taken six weeks to reach the crown. Perks et al. (2002), therefore, concluded that a feed-forward model of short-term stomatal response to soil drying, if based solely on the action of a chemical messenger from the roots, is not applicable in mature conifer trees because signal transmission is too slow.

Roots play a critical role in sensing water deficits. Under drought stress, roots continue to grow, whereas shoot growth stops (Wilkinson and Davies

2002). Under these conditions, roots accumulate ABA, which is necessary for sustained growth (Sharp et al. 1994, 2004a,b). It has been proposed that the role of ABA in controlling root and shoot growth was indirect, via ethylene. High ABA concentrations prevented ethylene accumulation and enabled growth, whereas low shoot ABA caused growth decline (Sharp et al. 2000; Sharp 2002). Although these results were obtained with maize, tomato, and other crops, they seem to be valid for trees as well. In a very detailed analysis of growth during the development of drought stress, it was found that radial stem growth was the most sensitive parameter, declining already before any decreases in plant water potential, stomatal conductance or other ecophysiological parameters were apparent (Bogeat-Triboulot, Fayyaz and Polle, unpublished). When drought stress increased further, shoot extension growth also decreased whereas root growth was affected only when drought stress had caused a severe decline in photosynthesis. This shows that soil water deficits have a diverse influence on different parts of a tree and that the response of the tree to drought follows a strict orchestration, whose underlying mechanism is not yet fully understood.

13.4.2 Drought Stress Requires Osmotic Adjustment

With decreasing soil water potential, water flux into the plant decreases, and if no compensatory measures are taken the plant wilts and dies. In order to ensure water supply, it is necessary that the plant adjusts its internal water potential to values below those occurring in the soil. The multiple signalling chains in different plant tissues, as outlined above, lead to adaptive mechanisms involving the synthesis of solutes, which are required for osmotic adjustment and which are therefore termed osmolytes. Increasing concentrations of solutes decrease plant water potential and enable maintenance of water uptake (Rathinasabapathi 2000). Since these compounds accumulate at high concentrations without disturbing cellular metabolism, they have also been called “compatible solutes”.

Compounds fulfilling these functions, as well as “osmoprotectants” which protect cells against osmotic shock by other mechanisms, belong to several chemical classes, such as polyols, amino acids, and quaternary amines, including mannitol, raffinose, galactinol, trehalose, glycine betaine, proline, etc. (Rathinasabapathi 2000). These metabolites increase in many species upon exposure to drought or other osmotic stresses (Hanson and Hitz 1982; Rhodes and Hanson 1993), and manipulation of their levels in transgenic plants resulted in some cases in increased stress tolerance (Tarczynski et al. 1993; Kishor et al. 1995; Pilon-Smits et al. 1995; Karakas et al. 1997; Sheveleva et al. 1997a; Kasuga et al. 1999; Nanjo et al. 1999; Huang et al. 2000; Garg et al. 2002; Sakamoto and Murata 2002; Taji et al. 2002, 2004; Vinocur and Altman 2005). Thus, many of these compounds have been discussed in the context of “osmotic adjustment”. However, true osmolytes are compounds

that accumulate to concentrations required to decrease the water potential of the plant below that of the soil, but in many cases this was not the case, proline being a prominent example (Blum et al. 1996). Most studies addressing this issue, including tree species, reported that drought caused very substantial increases in proline (Arndt et al. 2001; Watanabe et al. 2001; Peuke et al. 2002; Sofo et al. 2004). Manipulation of the biosynthetic pathway for proline, either directly via overexpression of Δ^1-pyrroline-5-carboxylate synthetase or indirectly by activation of calmodulin-mediated stress signalling, resulted in increased proline concentrations and the transgenic plants were more resistant against drought, salt, and cold (Kishor et al. 1995; Yoo et al. 2005). However, calculations showed that even tremendous increases in proline, such as reported, e.g., by Kishor et al. (1995), are not sufficient to contribute substantially to the adjustment of the osmotic pressure (Blum et al. 1996).

We have recently shown that this was also true in the salt-tolerant tree *P. euphratica* (Ottow et al. 2005). Upon salt stress, which imposed osmotic stress of about −1.6 MPa to plants tissues, *P. euphratica* showed more than 100-fold increases in proline rising from almost undetectable low concentrations to 3.5 μmol/g dry mass. The total increase in osmotically active substances was however much higher (300 μmol/g dry mass) and just sufficient for osmotic adjustment. It is, thus, immediately apparent that the overall concentrations of proline were too small to contribute significantly to osmotic adjustment (Ottow et al. 2005). Similar calculations have been conducted for mannitol showing that overexpression of this compound may only explain about 30–40% of the osmotic adjustment (Karakas et al. 1997). Nevertheless, plants accumulating this compound were more stress-resistant. Since compatible solutes also have free radical scavenging capacities (Shen et al. 1997a,b), it has been suggested that they may act to protect the plants against oxidative injury or may function in redox regulation (Shen et al. 1999). Another explanation for the protective function of these compounds is their activity as chemical chaperones by stabilizing proteins and preventing their unspecific unfolding and loss of function (Vinocur and Altman 2005).

We are not aware of any studies addressing drought resistance in trees by manipulating the levels of compatible solutes. However, recently Hu et al. (2005) showed that transgenic poplar overexpressing a mannitol-1-phosphate dehydrogenase gene (*mtl*D) contained increased concentrations of mannitol and were more tolerant to salt stress. It remained questionable whether salt tolerance was related to increased osmotic protection or to other effects of mannitol, since the increase in mannitol was more than compensated by the decreases in glucose (Hu et al. 2005). The trees showed a dwarfed appearance, which might have been caused by diversion of carbohydrate from growth to pathways of mannitol biosynthesis. It cannot be excluded that excess mannitol has toxic effects, similar to over-accumulation of sorbitol in tobacco (Sheveleva et al. 1997b). In this context it is notable that proline did not increase when Arabidopsis was simultaneously exposed to heat and drought (Rizhsky et al. 2004). Proline accumulation was toxic at higher

temperatures and was apparently replaced by sucrose for osmoprotection (Rizhsky et al. 2004). These results show that transgenic approaches to engineer drought tolerance directly via constitutive increase of osmolytes or osmoprotectants may be limited. It appears more promising to employ strategies for increasing drought tolerance by manipulation of the transcriptional control, as suggested by Zhang et al. (2004b) and to use drought-induced promoters that have low constitutive expression.

13.4.3 The Cells' Weapons to Prevent Drought-Induced Injury

Under drought stress, water becomes limiting, the stomata close and the plant must cope with excess light. Mechanisms to dissipate excess light include the xanthophyll cycle, photorespiration and the Mehler reaction. The Mehler reaction leads to the formation of reactive oxygen species (ROS) such as H_2O_2 and O_2^- and, thus, an increased need for ROS detoxification in the chloroplast (Polle 1996a). Drought-induced increases in superoxide and H_2O_2 by the thylakoid membranes have been documented (Bartoli et al. 1999). The extent of accumulation of these oxidants is determined by the capacity of the major redox buffers of the plant cell, ascorbate and glutathione, and the associated enzymes of antioxidant defence, i.e. superoxide dismutase, ascorbate peroxidase, glutathione reductase. Many studies in crop plants and tree species revealed that general enhancements of antioxidant defences accompanied prolonged exposure to water deficits (Smirnoff 1993; Polle 1996b). We have shown that not only the concentrations of protective metabolites or activities of antioxidant systems in leaves of trees species were important to prevent oxidative injury, but especially the responsiveness to such environmental challenges (Schwanz et al. 1996; Schwanz and Polle 2001). In tree species, the ability to adapt rapidly to oxidative threats declined with increasing leaf age, rendering older leaves more prone to oxidative injury (Polle et al. 2001).

Transformed plants with increased expression of antioxidant enzymes have been produced with the aim to increase drought tolerance. In particular, overexpression of either superoxide dismutase (SOD) or ascorbate peroxidase in the chloroplast (McKersie et al. 1996; Yan et al. 2003) has been shown to confer some, though moderate, degree of protection against water deficits. Overexpression of Fe-SOD in poplar chloroplasts was found to protect the photosynthetic electron transport system from over-reduction at low CO_2 partial pressures (Arisi et al. 1998), but soil drought was not tested. Given the relatively limited success of these approaches, it appears that the chloroplasts are either inherently well protected against oxidative stress or that the Mehler reaction does not play an important role under drought (Noctor et al. 2002).

Probably the most important mechanism for dissipation of excess electrons produced in drought-stressed leaves is provided by photorespiration. Transgenic plants with a diminished capacity for photorespiration became

more drought sensitive (Kozaki and Takeba 1996). While the operation of photorespiration may attenuate ROS production in the chloroplast, glycolate oxidation involves the obligatory production of H_2O_2 in the peroxisomes. Employing catalase-deficient tobacco plants, Willekens et al. (1997) showed that catalase was indispensable to prevent increased oxidative load in photosynthetic cells. Unexpectedly, transgenic poplar trees overexpressing glutamine synthetase were more resistant against drought stress than wild type controls and this was probably due to an increased capacity for photorespiration, as evident from a greater burst of CO_2 efflux in transgenic than in non-transgenic plants under sudden reduction in photosynthetic photon flux (El-Khatib et al. 2004). Under drought, the transgenic poplar showed higher photochemical quenching and higher light-adapted PSII yield than the non-transgenic plants. Under severe water stress, transgenic plants maintained higher expression of glutamine synthetase, glutamate synthetase and Rubisco as well as higher concentrations of chlorophyll and glycine than non-transgenic plants (El-Khatib et al. 2004). The sustained photosynthetic electron transport capacity in poplar overexpressing glutamine synthetase suggests that these plants had increased photorespiratory activity, and that this served as a protective sink for electrons from photosynthetic reaction centres (El-Khatib et al. 2004).

If ROS accumulate due to insufficient stress compensation, they may have signalling functions leading to ABA synthesis and increased protection (Kwak et al. 2003; Gechev and Hille 2005). This is normally the case at the onset of stress. However, unattended overaccumulation results in the formation of lipid peroxides, which are then converted into highly toxic aldehydes (Esterbauer et al. 1991). Analysing the mechanisms enabling resurrection plants to cope with cycles of drying and watering, Bartels' group (Bartels et al. 1990; Kirch et al. 2001) discovered that aldehyde dehydrogenase played a crucial role in desiccation tolerance. Since the presence of this class of enzymes was not confined to desiccation tolerant species, it was concluded that tolerance is not the result of an exclusive trait but caused by differences in gene regulation (Hilbricht et al. 2002). Overexpression of aldehyde dehydrogenase rendered plants more drought tolerant (Oberschall et al. 2000; Sunkar et al. 2003). In tree species, this approach has not yet been tested, but it appears to be promising for improving drought tolerance.

In addition to systems involved in detoxification and repair, plants also contain an array of proteins that function directly in the protection of proteins and membranes such as HSPs (heat shock proteins) and chaperones (Wang et al. 2004). HSPs and chaperones are usually small proteins, which accumulate under drought and other stress conditions. Initially, late embryogenic abundant proteins (LEA) were identified (Vierling 1991). It is now clear that some of these or related proteins act as chaperones and help to stabilize or refold other cellular proteins, thus preventing denaturation during stress. Despite their abundance in plants, our knowledge about their action is still very limited.

In tree species, molecular chaperones also seem to have important functions in abiotic stress acclimation (Vinocur and Altman 2005). In aspen,

a boiling-stable protein, SP1 (formerly referred to as BspA), was found to accumulate in response to water stress and abscisic acid application (Pelah et al. 1995). Subsequently, the *sp1* gene was cloned from aspen (*Populus tremula*) plants by immunoscreening an expression library using polyclonal antibodies against the SP1 protein (Wang et al. 2002). *sp1* encodes an exceptionally stable 12.4-kD hydrophilic protein, which forms a dodecamer and was found to accumulate following salt, cold, heat, and desiccation stress, but was not responsive to ABA (Wang et al. 2002). Preliminary evidence suggests that overexpression of SP1 mediates increased tolerance against osmotic stress imposed by salt (Barak, Vinocur and Altman, unpublished data). Recently, the three-dimensional structure of SP1 was determined, using X-ray crystallography (Dgany et al. 2004).

13.5 Profiling of Gene Expression and Protein Patterns: New Tools for Improving Drought Tolerance in Trees?

It is now clear that drought tolerance is a multi-genic trait. Thus, overexpression of a single gene is likely to act only in a limited metabolic window, and perhaps only under certain conditions. In order to understand the responses of plants, and of trees in particular, new genome- and proteome-wide analyses techniques enable us to develop new strategies to analyse the response to drought, and identify key targets to improve stress tolerance. Hundreds of drought responsive genes have been identified in numerous studies. However, not all of these studies have been conducted under conditions which are likely to yield meaningful results. If a stress is administered too abruptly or too strongly, it is unlikely that adaptive responses can be observed – instead the result of destruction is found. Rigorous review of studies dealing with drought-transcriptome-profiling in Arabidopsis and electronic analyses of the published data left Bray (2004) with a list of only 27 genes, which are likely to play a role in stress adaptation. This list excluded all those experiments, which were conducted under too harsh conditions. Three experiments were analysed, in each of which drought was induced in Arabidopsis in a different experimental set-up, either by simple drying on filter paper (Seki et al. 2001), or by application of impermeable solutes (Kreps et al. 2002) or by gradually developing soil drought (Kawaguchi et al. 2004). In the light of the previous discussion (see Sect. 13.2), it is not surprising that up regulation of the expression of aldehyde hydrogenase, dehydrins, LEA proteins, and phosphatases involved in signal transduction were observed (Table 13.1).

To find common drought-responsive candidate genes in Arabidopsis and trees, we have extended this analysis and compared the list of 27 genes (Bray 2004) with two studies on expression profiling in trees, i.e. pine (Watkinson et al. 2003) and poplar (Bogeat-Triboulot et al., unpublished). None of the

Table 13.1. Genes induced in common when expression patterns are compared between three different water-deficit stress conditions done in different laboratories for *Arabidopsis* (Seki et al. 2001; Kreps et al. 2002; Kawaguchi et al. 2004) as compiled by Bray (2004), pine (Watkinson et al. 2003) and poplar (Bogeat-Triboulot et al., unpublished). The genes are grouped by potential functional categories. Only genes analysed in leaves are compared

Gene name and description	Arabidopsis	Poplar	Pine
Metabolism			
Cinnamyl alcohol dehydrogenase	+	+	0
Aldehyde dehydrogenase, putative	+	0	+
Flavin-binding monooxygenase-like domain	+	0	0
β-Alanine-pyruvate aminotransferase, putative	+	0	0
Endochitinase	+	0	0
Asparagine synthase	0[a]	+	+
Sucrose synthase	0[a]	–/(+)	+
Thaumatin-like protein PR-5b precursor	0	–	+
Redox			
Thioredoxin H	0	–	+
Electron transport			
Alcohol dehydrogenase	0[a]	+	+
Transporters			
Mitochondrial dicarboxylate carrier protein	+	0	0
Amino acid/polyamine transporter, family II	+	0	0
Signal transduction			
PP2CA, protein phosphatase 2C	+	0	0
ABI1, protein phosphatase 2C	+	0	0
Transcription			
ATHB-7, homeobox-leucine zipper protein	+	0	0
RD26, transcription factor, NAM like	+	0	0
Lea14-A-like cotton (Class VI)	+	0	0
Dehydrin, COR47/rd17 (Class II)	+	+	0
LEA76-like Brassica napus (Class III)	+	–/(+)	0
LEA-like (Class I)	+	+	0
Dehydrin, RAB18 (Class II)	+	–	0
LTI65/RD29B; low-temperature-induced	+	0	0
65-kDa protein LTI78/COR78/RD29a; 78 kDa protein	+	0	+

[a] Genes with elevated expression level in Rizhsky et al. (2004)
Symbols indicate changes in transcript levels: + = increase, 0 = no change, – = decrease

genes found to be up regulated in the Arabidopsis list (Bray 2004) showed increased expression in both tree species under stress (Table 13.1). However, putative aldehyde dehydrogenases as well as a transcription factor (RD29a) were increased in both Arabidopsis and poplar, whereas cinnamyl alcohol dehydrogenase (CAD) expression was increased in both Arabidopsis and pine (Table 13.1). It should be noted, however, that recent evidence indicates that stress-induced genes with high homology to CADs actually did not use cinnamylaldehydes as their substrate and, therefore, should be re-termed as stress-related oxidoreductases (Jouanin, personal communication).

In a recent analysis of the drought-responsive transcriptome of Arabidopsis, the expression levels of alcohol dehydrogenase, sucrose synthase, and asparagine synthetase were increased (Rizhsky et al. 2004). The expression levels of these three genes were also up regulated during drought stress in pine and poplar, suggesting that they may represent key processes for drought adjustment. Increased alcohol dehydrogenase may be required to adjust mitochondrial electron transport. Sucrose synthetase might reflect increased turnover of carbohydrates, whereas the function of asparagine synthase in stress acclimation is unknown.

The development of drought stress is a dynamic process involving probably different acclimatory reactions at different stages of declining water potential. The static comparison of stress-responsive gene products is, therefore, expected to filter only prominent, persisting responses. In pine, transcriptional responses to drought were studied at higher time resolution involving mild (–1 MPa) and severe (–1.5 MPa) drought stress (Watkinson et al. 2003). Under mild drought stress up-regulation of LEA genes, phenylpropanoid metabolism related genes and genes for polyamine encoding enzymes were found, whereas the latter were decreased under severe drought stress. Using a slightly different strategy, Dubos et al. (2003) identified 28 transcripts that were increased in maritime pine exposed to drought stress. Those corresponding to proteins of known function confirmed that the main pathways involved in the osmotic stress response were photosynthesis, carbohydrate metabolism, cell wall synthesis, and plant defence. In three unrelated loblolly pine genotypes, 42 transcript levels (out of 6765) that significantly increased under drought resembled gene products which were suggested to be important for drought tolerance in other species, including dehydrins, endochitinases, cytochrome P450 enzymes, pathogenesis-related proteins and various late-embryogenesis abundant (LEA) genes (Lorenz et al. 2005).

An intriguing question is whether increased levels of transcripts are also translated into increased concentrations of products, i.e. functional proteins and metabolites? Currently, only a few proteome analyses have been conducted and it is therefore premature to come up with final conclusions. For example, an analysis of the proteome of mitochondria from drought stressed peas showed induction of HSP (Taylor et al. 2005), supporting interpretations from previous transcriptional analyses. Moreover, these authors showed that

the mitochondrial electron transport chain was not affected (Taylor et al. 2005). Transcriptome analyses implied stimulation of the phenylpropanoid metabolism (see above), but the proteome of maize leaves showed reductions in caffeate-*o*-methyl transferase and a decrease in lignin (Vincent et al. 2005). However, it would obviously be necessary to analyse the response in the same plant at the same stress level in order to be conclusive about the role of secondary metabolism in drought adaptation.

In spruce the needle proteome was analysed under mild drought stress (Blödner 2005), not affecting any ecophysiological parameter like water potential or photosynthetic electron transport. Under these conditions, proteins of the water oxidising complex (OEE1 and OEE2) significantly diminished and fragments of Rubisco appeared, suggesting that degradation was already taking place (Blödner 2005). Furthermore, a protein with yet unknown functions was up regulated (Blödner 2005). These data suggest that the chloroplast and its proteins related to photosynthetic electron transport and consumption may be more drought sensitive than the mitochondrial electron transport chain.

These brief spotlights on transcriptional and proteomic analyses show that these novel methods are useful to identify stress-responsive genes and thus will be helpful to elucidate signalling chains orchestrating plant response to drought. However, to understand whether a signal will lead to a new stage of cellular homeostasis, it is necessary to combine transcriptional analyses with proteomic and metabolomic analyses. It is also important to choose realistic conditions for exposure to stress. The recent observation that drought and heat – two factors that occur normally together in nature – may evoke different stress patterns compared with drought alone is important and calls for analyses conducted under field conditions.

13.6 Conclusions

Currently, most tree-improvement programs are based on the management of genetic resources, including the selection of superior clones from existing forests, the conservation of genetic variability, partially controlled propagation and classical breeding for desired traits. Although molecular breeding is routine in agriculture, and numerous agrobiotechnological companies are producing many new genetically engineered field crops, vegetables and ornamental plants, forest tree species have been left far behind (Tzfira et al. 1998). Plant transformation techniques, gene isolation and characterization are no longer serious obstacles, and so forest trees are becoming an attractive target for genetic engineering and molecular breeding in the twenty-first century, even though various constraints need to be overcome. Different tools are now available to transform plants genetically, and the most commonly used – *Agrobacterium* and particle bombardment – have been extensively

reviewed (Teichmann and Polle 2006). Early reports on the genetic transformation of forest trees focused on *Populus* species (Leplé et al. 1992). Even today, *Populus* remains the principal genetically transformed tree species, both as a model system and for practical reasons. Transformation of other economically relevant tree species such as pine, spruce or *Eucalyptus* has been described but is still confined to specialized laboratories. In this area technical advance is required.

Furthermore, the ecological consequences of transformation need to be taken into account. During their perennial life cycle, forest trees must adapt to seasonal climatic changes and to a wide range of pests and abiotic stresses. Consequently, tree populations exhibit high diversity, reflected in many ecotypes; there is, therefore, a need to transform different ecotypes for stable expression of the transgenes through cycles of environmental changes. Additionally, the long life cycle of forest trees calls for stability of the transgenes over several years. As the common constitutive promoters are silenced in many annual transgenic plants, unique constructs with more suitable promoters, preferably of tree origin, are likely to be required for the long-term expression of foreign genes in forest trees. Nevertheless, it should be noted that homologous promoters could also be silenced in transgenic trees by positional effects or other epigenetic processes.

Since stress tolerance is a multigenic trait, the genetic engineering for drought tolerance requires a deeper understanding of the key mechanisms involved in drought resistance. To advance this knowledge integrated approaches studying drought tolerance with ecophysiological, molecular, metabolomic, and proteomic methods are urgently needed. To unravel the mechanisms of drought tolerance in trees beyond common mechanisms also present in herbaceous plants, it will be necessary to devise experiments addressing the unique characteristics of tree species. As trees are perennial and exist for decades, different stages of their life cycle can be distinguished. The first, and probably most vulnerable phase with respect to drought, is the establishment of the seedlings. An important question is whether seedlings and mature trees show similar or different regulation of their responses to drought. The development of certain morphological structures is also important for drought adaptation. Many trees develop a specific root and xylem system, which favours drought resistance, e.g., roots that explore deep soil zones and a xylem resistant to cavitation. Experiments addressing such adaptive traits are missing to date.

The knowledge of how trees adapt to drought is of interest not only for academic but also for various practical reasons. One is that large-scale deforestation results in changes in the microclimatic conditions, generally causing decreased water availability and, thus, requires afforestation with trees adapted to severe environmental stress. Another important reason is that the demand of renewable resources including wood will increase because of the increasing world population and, thus, requires an extension of areas used for silviculture. Since fertile soils amenable for agriculture will not be

available for this purpose, the breeding of tree species able to persist under arid or semi-arid conditions is necessary. Furthermore, significant changes in precipitation patterns are predicted in temperate regions with currently high precipitation during the growth phase. Current forest management and cultivation strategies are not in agreement with the expected future climatic changes, and thus require the introduction of more stress tolerant ecotypes or novel engineered tree species to minimise the risk.

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Part D Biosafety Issues

14 Genome Instability in Woody Plants Derived from Genetic Engineering

HANS HOENICKA AND MATTHIAS FLADUNG

14.1 Introduction

Constant genome change is the driving force of evolution. Higher plants display remarkable phenotypic plasticity during their development. Unlike higher animals, in which virtually all organs of the adult are present in the embryo at least in rudimentary form, the plant embryo contains only the initial cells of its organs. Continual organogenesis offers plants the opportunity to produce a range of phenotypes from a single genotype (Walbot 1996). Day length and light quantity, water and nutrient status, density of herbivores, pathogens and many other environmental factors can elicit genome instability, and lead to changes in plant phenotype and ecological fitness.

The release of plants derived from genetic engineering (GE) has raised questions about possible interactions of transgenic sequences with the plant genome. In this review we present information available about genome instability in transgenic and non-transgenic plants in order to give an holistic frame to biosafety discussion on woody plants derived from GE. Initial results on risk assessment of genome instability in genetic modified (GM) woody plants are also presented.

14.2 Genetic Engineering of Woody Plants

GE has mainly been applied for breeding of crop plant species. The utilization of GE for improvement of transgenic woody plants lags still far behind. Even so, more than 35 different woody plant species have been genetically modified through GE worldwide (Mullin and Bertrand 1998; Petri and Burgos 2005). The first confirmed record of a release of a GM woody plant species is that of a poplar trial in Ghent, Belgium in 1988 (van Frankenhuyzen and Beardmore 2004). Most releases of transgenic woody plants have occurred in the USA, but there are also some reports in the European Union, New Zealand and China. There are also commercial plantations of GM Poplar (Wang et al. 2003) and Papaya (Gonsalves 1998; Chiang et al. 2001) in China and the US, respectively.

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Tree Transgenesis: Recent Developments

The promising prospects offered by GE has promoted efforts to develop molecular breeding methods for woody plants. The genetic transformation of plants has some advantages in comparison to conventional breeding: (a) genes from virtually any organism can be introduced in the breeding process, thus broadening the range of genes available outside the current boundaries of the genus, (b) individual genotypes can be improved for one or a small number of well defined traits while preserving the rest of the genome intact, and (c) GE is the only method available which could allow accelerated breeding of forest tree species at rates comparable to that achieved with crop plants. This latter advantage is particularly important as breeding of forest trees has been hampered by the long time they require to enter into the reproductive phase.

However, GE has been the subject of considerable controversy, with concerns raised mainly from ecological and ethical arguments. This controversy has slowed the broad use of GE in agriculture and forestry. The following biosafety issues have been discussed: (1) genome instability, (2) vertical gene transfer, (3) horizontal gene transfer, (4) impact on non-target organisms, (5) invasive potential of genetic modified plants, and (6) unpredictable pleiotropic effects due to random insertion of foreign genes (Hoenicka and Fladung 2006).

14.3 Genome Instability in Plants

Environmental factors can elicit changes in the plant genome, which can broadly be classified as either abiotic (physical and chemical agents) or biotic (living organisms such as pathogens) (Kovalchuk et al. 2001). Many different abiotic factors such as ionizing and UV radiation, various chemicals, or even changes in sodium chloride concentration and light intensity were shown to destabilize the plant genome (Lebel et al. 1993; Puchta et al. 1995; Kovalchuk et al. 1998; Ries et al. 2000; Lucht et al. 2002; Kovalchuk et al. 2003; Filkowsky et al. 2004). Besides exposure to various physical and chemical factors, plants are persistently exposed to diverse biotic stresses, including bacteria, viruses, fungi, nematodes, and insects which can also destabilize the plant genome (Filkowski et al. 2004). Infection of tobacco plants with tobacco mosaic virus and oilseed rape mosaic virus was shown to induce a threefold increase in homologous DNA recombination compared to non-infected tissues (Dong 2004). Exposure to a viral pathogen led to increased transposon excision in the progeny of virus-infected maize plants (Johns et al. 1985).

14.3.1 Genome Instability Caused by Viruses and Repetitive Elements in Plants

Repetitive sequences, like DNA transposable elements and retroelements, terminal repeat sequences and tandem repeat sequences, make up large portions of plant and animal genomes (Jakowtisch et al. 1999; Kumar and

Bennetzen 1999; Matzke et al. 2000). In some plant species, e.g. maize, they constitute up to 90% of the genome (San Miguel et al. 1996; Kazazian 2004). Transposable elements can generate mutations by inserting within or near genes (Kumar and Bennetzen 1999). On the basis of mechanisms of their transposition, transposable elements can be divided into two classes: retrotransposons (transposable elements class I), which proliferate via reverse transcription, and DNA transposons which are transposed without RNA intermediates. The DNA transposable elements *Ac*, *Tam1*, *En/Spm* transpose by an excision/repair mechanism (transposable elements class II) and usually do not greatly increase plant genome size (Kunze et al. 1997). In contrast, retrotransposon-induced mutations increase genome size because they transpose via replication, and thus the sequence at the original insertion site is retained (Kumar and Bennetzen 1999). A recently identified class of DNA transposable element (also called transposable elements class III), with similarities to the bacterial rolling-circle transposons, transpose by a rolling circle replication mechanism similar to that found in some plasmids, single stranded bacteriophages, and plant geminiviruses (Le et al. 2000; Feschotte and Wessler 2001; Kapitonov and Jurka 2001; Wright et al. 2003).

Environmental conditions and many stress factors activate retrotransposons and other mobile elements (Grandbastien 1998). These elements induce spontaneous mutations responsible for phenotypic changes in *Ipomoea* spp. flowers (Hoshino et al. 2003), maize leaves (Vignols et al. 1992), grape skin (Kobayashi et al. 2004), maize seeds (Marillonnet and Wessler 1997) and in vitro cultures of different plants (reviewed in Grandbastien 1998). For Drosophila, in which DNA methylation does not occur, 80% of spontaneous mutations are caused by the insertion of retrotransposons (Green 1988). In plants, were most retrotransposons are methylated, this proportion is much lower (Wessler 1996).

Transposable elements are a major source of genetic variation that ranges from gross chromosomal alterations up to very fine tuning of the expression of cellular genes (Kunze et al. 1997). This, together with the observation that transposons are activated by stress and environmental factors, led to the hypothesis that transposable elements are involved in host adaptation to environmental changes (McClintock 1984; Wessler 1996; Grandbastien 1998). In particular, through modifications of gene regulation, transposable elements have been proposed as major factors in macroevolution (McDonald 1990). The question as to whether transposable elements are selfish parasitic sequences or pacemakers of evolution is still controversial (Kunze et al. 1997).

Viral DNA sequences can become incorporated in host genomes (Tanne and Sela 2005). Integrated (endogenous) viral sequences are familiar components of bacterial and animal genomes (Matzke et al. 2004). About 8% of the human genome, for example, is derived from retroviruses (Hughes and Coffin 2001). Many plants contain already integrated sequences from both

types of plant DNA viruses, the single-stranded DNA geminiviruses and the double stranded DNA pararetroviruses (Richerd-Poggeler and Shepherd 1997; Harper et al. 2002; Gregor et al. 2004; Murad et al. 2004), and retrovirus-like elements (Vicient et al. 2001; Wright and Voylas 2002).

14.3.2 Polyploidy

Grant (1981) described polyploidy as 'the formation of a higher chromosome number by the addition of extra whole chromosome sets present in one or more ancestral organisms'. This classical definition is still applied today, although recent research indicates that most organisms have polyploidy somewhere in their evolutionary history (reviewed in Soltis et al. 2003). Several mechanisms may lead to polyploidy in plants: (1) somatic doubling, at the zygotic embryonic, or meristems of a plant (Jørgensen 1928; d'Amato 1952, 1964), (2) gametic "non-reduction" or "meiotic nuclear restitution" during micro- or megasporogenesis, resulting in unreduced gametes (Bretagnolle and Thompson 1995), (3) polyspermy, the fertilization of an egg by more than one sperm nucleus (Hagerup 1947; Vigfússon 1970), and (4) interspecific hybridisations – there is a general agreement that unreduced gametes are the major mechanism of polyploid formation (Ramsey and Schemske 1998).

A major discovery was the extent and rapidity of genome reorganization in polyploids (Soltis et al. 2003). A diversity of molecular approaches, including chromosome painting methods (GISP, FISH), genetic mapping, and comparative genetics provide evidence for both intra- and intergenomic reorganization of polyploid genomes (Soltis and Soltis 1993, 1999; Wendel 2000). Genomic downsizing (Vicient et al. 1999; Hancock 2002), gene silencing (Comai 2000), novel gene expression (Comai 2000; Osborn et al. 2003) and activation of transposable elements (Matzke et al. 1999) have been detected in polyploid plants.

Several researchers have found that $2n$ pollen production is stimulated by environmental factors such as temperature, herbivory, wounding, and water and nutrient stress (Ramsey and Schemske 1998). Temperature, and especially variation in temperature, have particularly great effects (McHale 1983; Ramsey and Schemske 1998). Corn plants exposed to 40 °C approximately 24 h after pollination produced 1.8% tetraploid and 0.8% octoploid seedlings (Randolph 1932). Many of the environmental factors known to influence $2n$ gamete production are experienced by plants in their natural habitats. The high incidence of polyploidy at high latitudes, high altitudes, and recently glaciated areas may be related to the tendency of harsh environmental conditions to induce $2n$ gametes and polyploid formation (Sax 1936).

Autopolyploids arise within single populations or between ecotypes of a single species, whereas allopolyploids are derived from interspecific

hybrids (Gottschalk 1978; Ramsey and Schemske 2002). Allopolyploids are frequent in nature, fertile, well adapted and genetically stable (Madlung et al. 2005). Allopolyploids of recent origin, however, commonly display phenotypic instability, low fertility, low embryonic viability, severe meiotic irregularities involving poor chromosome pairing and non-disjunction (Soltis and Soltis 1995; Ramsey and Schemske 1998; Comai 2000; Schranz and Osborn 2000). The mean frequency of 2*n* gametes found in hybrids (27.52%) was nearly 50-fold higher than that in nonhybrids (0.56%) and might be in some hybrids even higher (Ramsey and Schemske 1998). Presumably, the instabilities that are manifested by recent formed allopolyploids are mitigated in the process of evolutionary adaptation that gives rise to stable species (Comai 2003).

Three kinds of genomic changes might explain genome instability in allopolyploids: (1) epigenetic alterations leading to activation or repression of gene expression, (2) activation of transposable elements, and (3) large-scale chromosomal rearrangements (Madlung et al. 2005). Synthetic allotetraploids of *Arabidopsis thaliana* displayed instability of selected transposons, genomic rearrangements, and chromosomal abnormalities involving the formation of bridges and chromosomal breaks (Madlung et al. 2005).

14.4 Genome Instability in Transgenic Plants

The theoretical molecular basis for plant transformation is the exact insertion of a single transgene in the recipient genome without any further genomic disruption. However, in practice a number of genomic variations have been reported following: (a) tissue culture procedures, (b) gene transfer methods, and (c) foreign DNA insertion (including the T-DNA itself but also additional DNA like bacterial plasmid or genomic sequences).

14.4.1 Somaclonal Variation

Epigenetic or genetic changes observed in plants derived from in vitro cultures have been called somaclonal variation (Cullis and Kolodynska 1975; Cullis and Cleary 1986; Cullis 1990; reviewed in Kaeppler et al. 2000, Larkin and Scowcroft 1981 and Phillips et al. 1994). Somaclonal variation has been reported in poplar (Saieed et al. 1994a,b; Wang et al. 1996), spruce (Tremblay et al. 1999) and other woody plants derived from tissue cultures (Martins et al. 2004; Rodriguez Lopez et al. 2004). *Agrobacterium*-mediated gene transfer by floral dipping, which does not require an in vitro culture phase, avoids the undesired occurrence of somaclonal variation (Labra et al. 2004). The application of the floral dipping method towards the production of transformed woody plants is still very limited, due to lacking protocols and difficulties for using this approach with woody plants.

14.4.2 Molecular Marker Analysis of Genome Instability in Transgenic Plants

DNA polymorphism analyses have been used to compare the genomes of non-transformed plants with those of transgenic plants. In a comprehensive study with transgenic rice produced by different transformation procedures, the authors conclude that none of the rice transformation protocols avoid genomic changes (Labra et al. 2001), but they are lower following *Agrobacterium*-based transformation protocols. Transgenic sugarcane populations produced by *A. tumefaciens* infection were investigated for the presence of genomic variability using AFLP-profiling (Carmona et al. 2005). The results presented in this study clearly showed that these transgenic sugarcane plants revealed limited but detectable genomic changes and that, on average, these changes occur at the same rate in plant populations carrying different transgenes.

A similar approach was initiated by Fladung and Hoenicka for *Populus*-aspen transformed with *A. tumefaciens* (unpublished). One non-transformed control line Esch5 and four independent transgenic lines carrying the 35S-*rolC* gene construct (Fladung et al. 1997) were included in the study. In respect to the structure of the T-DNA insertion locus, integrated T-DNA borders as well as T-DNA-flanking genomic sequences in the four transgenic lines were previously described (Fladung 1999; Kumar and Fladung 2001, 2002). Three of these transgenic lines contained one single copy of the transgene (Esch5:35S-*rolC*) while the fourth line harboured an inverted repeat resulting in morphological instability (Esch5:35S-*rolC*-1) (Fladung 1999).

In the AFLP study with the 5 aspen lines, in total, 30 primer-enzyme combinations were tested from which 26 combinations revealed reproducible AFLP patterns (Fladung and Hoenicka, unpublished). Each primer-enzyme combination was repeated at least three times and only those bands which showed reproducible presence/absence patterns were counted. In total, 889 AFLP-bands were obtained for the 26 primer-enzyme combinations, ranging from 5 to 95 bands. From these 889 bands, only three variations belonging to three different primer-enzyme combinations were detected. Two of these variations were detected in two different transgenic lines and one in the control line. The remaining 886 bands were common in the non-transgenic control and in the four transgenic aspen lines. These results indicate that 35S-*rolC* transgenic aspen when compared to non-transgenic control plants show a high genomic stability. The rarely observed changes are possibly due to somaclonal variations or T-DNA integration changes.

14.4.3 Transgene Silencing

Many papers on transgenic annual plants show that expression of transgenes is less stable than it had originally been thought, and may vary between

transformants carrying the same number of foreign genes (Napoli et al. 1990; van der Krol et al. 1990; Fladung 1999; Bastar et al. 2004; Han et al. 2004). Experimental evidence proves that the mechanism of silencing plays a vital role in biological processes such as plant defence and plant development (Hunter and Poething 2003; Lu et al. 2003). Most such events reported a fall into the class of homology-dependent gene silencing, which involves mechanisms that function at the transcriptional and posttranscriptional level of transgenes (Jorgensen 1992, 1995; Matzke and Matzke 1993, 1995; Paszkowski 1994; Meyer 1995; Filipowicz and Hohn 1996; Stam et al. 1997). In addition, it is assumed that flanking plant DNA sequences and the nature of the integration site can influence transgene activity (Peach and Velten 1991; Kumar and Fladung 2001).

The stability of transgene expression has a decisive influence on the efficiency of strategies for biological confinement of transgenic plants. For example, in trees, strategies are currently under evaluation to avoid vertical gene transfer (Meilan et al. 2001; Hoenicka and Fladung 2003; Skinner et al. 2003; Fladung and Hoenicka 2004) by using sterility genes as *barnase* (Mariani et al. 1990) or *stilbene synthase* (Fisher et al. 1997). Gene silencing of these genes would result in crossings of transgenic woody plants with their natural relatives. Such instability represents an even higher risk factor in transgenic woody plants than in annual plants, because of their prolonged lifetime.

14.4.4 Structure of T-DNA Insertion Locus

Several groups have investigated the structure of genomic/T-DNA junctions in transgenic plants derived from *Agrobacterium*-mediated or biolistic transformation (Gheysen et al. 1991; Mayerhofer et al. 1991; Ehrlich et al. 1993; Kohli et al. 1999; Kumar and Fladung 2001). All investigations came to the same conclusion that integration occurs in both cases by illegitimate recombination. The vast majority of T-DNA insertion events also seem to include small or large genomic DNA disruptions and insertions of superfluous DNA (Forsbach et al. 2003; Makarevitch et al. 2003). There has been one large-scale study of the mutations created at insertion events containing single T-DNA inserts (Forsbach et al. 2003). In this study of 112 single-copy T-DNA insertions events in *A. thaliana*, most of the T-DNA insertions resulted in small (1–100 base pair) deletions of plant DNA at the insertion site. However, for 24 of the 112 transgenic lines there was evidence for a large-scale rearrangement of plant genomic DNA at the insertion site. Forsbach et al. (2003) found that 8 of 112 single copy T-DNA insertion events also had insertions of superfluous plasmid or T-DNA sequences. Insertion of T-DNA into plant cells occurs at random into an active DNA sequence. If T-DNA integration occurs inside a gene exon, generating a mutation (insertion-site mutation), transcription process of the gene is disturbed.

14.4.5 Recombination Between Transgenic Sequences, Viruses and Repetitive Elements

The potential for generation of new viruses through recombination applies to transgenic and non-transgenic plants. In plants infected with several viruses, the encapsidation of a virus genome with the coat protein of another virus (heteroencapsidation or heterologous encapsidation) has been regarded as an important element in the biosafety discussion (Hull 1998).

Recombination is considered to play a major role as a driving force in virus variability and thus in virus evolution (Aaziz and Tepfer 1999). Viral RNA replication is characterized by a high mutation rate due to the lack of proof-reading-repair of viral RNA-dependent RNA polymerases (Holland and Domingo 1998). RNA recombination occurs between closely related RNA molecules, but also between dissimilar RNAs (Lai 1992; Nagy and Bujarski 1993; Falk and Bruening 1994). The possibility of recombination between viral transgene transcripts and RNAs from field viruses infecting transgenic plants has been regarded as a major concern (de Zoeten 1991; Allison et al. 1996; Robinson 1996; Aaziz and Tepfer 1999; Martelli 2001; Tepfer 2002; Vigne et al. 2004). Resulting recombinant viruses may have similar characteristics to the parental lineages or new biological properties such as changes in vector specificity, expanded host range (Schoelz and Wintermantel 1993; Wintermantel and Schoelz 1996) and increased pathogenicity (Rubio et al. 1999).

Viruses have many opportunities to recombine with other viruses (Worobey and Holmes 1999) but also with non-transgenic host RNA (Monroe and Schlesinger 1983; Meyers et al. 1989; Mayo and Jolly 1991; Sano et al. 1992; Charini et al. 1994). Defective plant viruses, including such mutants of the CaMV (Gal et al. 1992; Schoelz and Wintermantel 1993), have been observed to repair their genomes through recombination with transgene transcripts of missing genes (present in T-DNA integrated in host plant) (Gal et al. 1992; Greene and Allison 1994; Gal-On et al. 1998; Borja et al. 1999; Rubio et al. 1999).

Retrotransposons also represent an important risk factor in both transgenic and non-transgenic plants. Long-terminal repeat (LTR) retrotransposons are strikingly similar to retroviruses in genomic organization and their coding sequences (Kumar and Bennetzen 1999). However, the lack of sequences encoding the envelope (ENV) protein restrict the retrotransposon replication cycle to the cell of the host genome. Phylogenetic analyses of retroviral reverse transcriptases suggest that LTR bearing retrotransposable elements can acquire additional open-reading frames that can enable them to mediate infection (Xiong and Eickbush 1990). This process and the acquisition of an envelope *env* gene is best documented in the origin of the vertebrate and invertebrate retroviruses (Malik et al. 2000). The origin of *env*-like genes is unclear. However, there is very plausible evidence that in some cases retrotransposon acquired and *env* gene from a viral source (Malik et al. 2000). The ty3-gypsy clade contains at least three putative instances of *env*

acquisition: the insect gypsy-like elements (Song et al. 1994), the plant *Athila*-like elements (Wright and Voytas 1998, 2002), and the *Osvaldo* element from *Drosophila buzzatii* (Pantazidis et al. 1999). The nematode *Cer* elements acquired their *env* gene from phleboviruses and the *Tas* element may have acquired it from a Herpesvirus-like ancestor (Malik et al. 2000). LTR-retrotransposons have been found inserted into baculovirus genomes (Malik et al. 2000), e.g. the TED retrotransposon found in the genome of associated *Autographa californica nucleopolyhedrosis virus* (ACNV) (Friesen and Niessen 1990). Thus, LTR retrotransposons can insert into viral genome, from which it has been suggested they obtained their *env* gene (Malik et al. 2000).

One of the characteristic features of transposable elements has been their spectacular ability to undergo cross-species horizontal transfers (Malik et al. 2000). This has been best documented in the case of the DNA-mediated elements, *P* and *Mariner* (Clark et al. 1994; Robertson 1997), but it is also true for LTR-retrotransposons (Jordan et al. 1999). The mechanism for horizontal transposon transfer still remains to be established. One likely scenario is that a transposable element could insert from the host genome into an associated DNA-based viral genome, which can subsequently infect another host species (Malik et al. 2000). In the case of RNA viruses, the retrotransposable element could simply be co-packaged within the viral capsid. Acquisition of an *env* gene, on the other hand, releases the retrotransposon from relying on another vector for jumping into different hosts, increasing the probability of cross-species transfer (Malik et al. 2000). The opportunism, shown by LTR retrotransposons in acquiring an *env* gene from another infectious agent, presents a general paradigm in which, any LTR retrotransposon can become a virus (Malik et al. 2000).

Transgene rearrangements have been detected both in lines obtained through *Agrobacterium*-mediated transformation (Deroles and Gardner 1988) as well as from particle bombardment (Register et al. 1994). The use of 35S promoter, from the pararetrovirus Cauliflower Mosaic Virus (CaMV), in most transgenic plants has been questioned for its viral origin and high recombination potential during the transformation process (Kohli et al. 1999). However, the 35S promoter has been proposed to represent a lower risk factor than viruses, endogene retrovirus/pararetrovirus and retrotransposons present in all plants (Hull et al. 2000; Matzke et al. 2000; Tepfer 2002). In fact, there is no evidence that the 35S promoter is mobile, in the way that a transposon can be (Tepfer 2002).

14.5 Transgene Stability in Woody Plants

Most research on risk assessment related to transgene instability in transgenic plants has been done mainly on annual, herbaceous species. However,

there is a very small number of publications available dealing with risk-related issues of transgenic woody plants.

14.5.1 Instability of Transgene Expression

14.5.1.1 Populus *spp.*

Analysis of GUS expression of 35S:*uidA* transgenic poplar grown in a field trial in France revealed that all transgenic plant lines showed stable expression of the transgene (Pilate et al. 1997). Hawkins et al. (2003) evaluated transgene expression in a hybrid poplar (*Populus tremula* × *P. alba*) clone transformed with constructs carrying a reporter gene (*uidA*) under the control of either a constitutive or a vascular-specific promoter. Analyses of transgene expression by GUS fluorometry and histochemistry was performed on several hundred trees, originating from different transgenic lines, grown under in vitro, greenhouse and field conditions. While important variations in expression levels occurred, the transgene appeared to be stably expressed throughout a six-year period. A similar result was reported for hundreds of different poplar transformants carrying various gene constructs and tested under field conditions (Strauss et al. 2004). Even when 35S::*uidA* and rbcS::*uidA* transgenic trees are treated with stress conditions (high temperature, UV-light), no stress-related transgene silencing could be observed for poplar (InfoNet-Umwelt SH 2004).

Silencing in the 35S::*uidA* transgenic poplar was detected only for lines which were probably silenced from the beginning shortly after the transformation process (Hawkins et al. 2003; InfoNet-Umwelt SH 2004). However, due to the destructive nature of the GUS activity test or other enzyme measurement procedures, only a small part of the plant at a given time can be screened with respect to transgene stability. As shown by Kumar and Fladung (2001) and Fladung and Kumar (2002), inactivation of the phenotypic marker gene construct 35S::*rolC* is a very rare event and occurs in an unpredictable manner. Thus, transgene silencing can happen at a single branch of a single plant among a high number of clonal ramets, and in the next year disappears in the same shoot (Fladung and Kumar 2002). Such silencing events remain undetectable with destructive reporter genes and can only be monitored when non-destructive reporter gene assays are being used. However, there is no evidence that expression of transgenes in plants under vegetative propagation is more variable than expression of most endogenes (Strauss et al. 2004).

Silencing was attributed to the different transgene copies organised either at two or more integration loci or as transgene repeat at one locus (Fladung 1999; Fladung and Kumar 2002; Kumar and Fladung 2001, 2002; Hawkins et al. 2003), or position effect including flanking genomic sequences (Kumar and Fladung 2001, 2002), or both (Fladung and Kumar 2002). Occurrence of a transgene repeat is often accompanied by methylation of the promoter

and/or the transgene (Kumar and Fladung 2000). However, not every transgenic line harbouring two T-DNA copies in repeat form is consequently silenced. Two 35S::*uidA* transgenic poplar lines produced in our laboratory, characterized by the presence of T-DNA repeats, revealed GUS expression over a period of seven years in plants cultivated either under greenhouse or in vitro conditions. It remains unknown so far whether these lines are "insensitive" to repeat-related transgene inactivation, or silencing has already occurred but it was not detected due to a low sensibility of the reporter gene used, or silencing of the transgene may happen some time in the future. Nevertheless, transgenic lines containing more than one T-DNA copy can easily be detected by simple molecular methods (Kumar and Fladung 2000) and subsequently discarded from the breeding process.

*14.5.1.2 Citrange (*Citrus sinensis *L.* Osbeck × Poncirus trifoliata *L. Raf.)*

Cervera et al. (2000) studied transgenic plants transformed with 35S::*uidA* by means of histological and fluorometric GUS analyses over a period of four to five years. Histological GUS analysis of transgenic plants showed conserved GUS expression levels during the whole period of study and no cases of a drastic variation in the transgene activity. However, fluorometric GUS assays showed in a same transgenic line variations of transgene expression of up to 40% in different seasons. ELISA assays performed to detect activity of the marker gene *nptII* showed a variable transgene expression, ranging from 1.1 to 6.7 ng of NPTII protein/mg of total protein, but in all cases it was significantly different from values obtained in the controls. Fluctuations were attributed to the developmental and physiological state of the plants.

A high variability of transgene expression and integration patterns was detected among the transgenic plants. Where T-DNA was inserted at a single locus, transformants displayed either high or low GUS activity. Plants which had integrated more than three transgene copies showed a level of GUS activity lower than 8 pmol MU/min per μg of total protein in all cases and could be considered as plants with silenced GUS activity.

*14.5.1.3 Spruce (*Picea mariana, P. glauca, P. abies*)*

Klimaszewska et al. (2003) reported a wide variability in expression of the *uidA* gene for *Picea mariana* translines produced by particle bombardment, with means per line ranging from 4.2 to 188.9 nmol 4-MU/min per mg protein. The highly GUS expressing lines always contained more than one copy of the transgene. Lower GUS expression were found in lines that integrated one to many *uidA* gene copies. It seems that there is no correlation between copy number and expression level or gene silencing in spruce.

GUS expression in *P. glauca* translines and *P. abies* ranged from 5.7 to 59.0 nmol 4-MU/min per mg protein and from 1.2 to 26.2 nmol 4-MU/min per mg protein, respectively. Correlation between the level of expression and the copy number of transgene could not be established for both species due to the very low number of lines carrying more than one copy of the transgene.

14.5.1.4 Pinus radiata

Wagner et al. (2005) induced gene silencing in transgenic lines of this gymnosperm species. A biolistic transformation procedure was used to transform embryogenic *Pinus radiata* tissue with constructs containing the *Zea mays* UBII (ubiquitin)-promoter followed by the *P. radiata CAD* (cinnamyl alcohol dehydrogenase) cDNA in sense, anti-sense orientation or in the form of an inverted-repeat. The effect of the different constructs on silencing the endogenous CAD gene was monitored in embryogenic tissue and somatic seedlings of 28 *P. radiata* transclones. A high proportion of transclones containing a *CAD* sense or antisense construct revealed a *CAD* over expression phenotype in embryogenic tissue compared to non-transformed control tissue. Quantitative CAD measurements demonstrated that the construct containing an inverted-repeat of the *CAD* cDNA was most efficient in triggering gene silencing in *P. radiata*. Northern hybridisation experiments with silenced transclones revealed that reduced CAD activities were the result of reduced steady state levels of the targeted *CAD* mRNA.

*14.5.1.5 Apple (*Malus *spp.)*

James et al. (1996) reported stable expression of both nopaline synthase (*nos*) and the cotransferred gene neomycin phosphotransferase (*nptII*) transgenes in the flesh of apple fruit and vegetative tissues from seven-year-old greenhouse plants. The very low levels of proteins in ripe transgenic apple fruit prevented the detection of NPTII in crude buffered extracts. When measurements were made on six-week-old auxin induced callus grown from transgenic or control flesh it was possible to detect NPTII protein (8–11 ng/mg protein) in the GM derived callus, but not in the control. Leaf material from the transgenic plants contained 12–1.5 ng NPTII/mg protein whereas untransformed plants had no detectable protein.

14.5.2 Recombination Between Transgenic and Virus DNA/RNA

*14.5.2.1 Grapevine (*Vitis *spp. L)*

Grapevine fanleaf virus (GFLV) is responsible for an important and widespread degeneration of grapevine (for details see Chap. 9), including fan-like

distortions of leaves and chlorotic yellowing as ringspots, vein banding, and mottling or mosaic patterns (Vigne et al. 2004). To investigate whether transgenic grapevines expressing the CP gene of GFLV assist the emergence of viable GFLV recombinants, Vigne et al. (2004) compared the molecular variability of the CP gene of GFLV isolates challenging transgenic and non-transgenic grapevines. Test plants consisted of non-transgenic scions grafted onto transgenic and non-transgenic rootstocks that were exposed to nematode-mediated GFLV transmission in two selected field settings over a three-year period. The CP gene of challenging GFLV isolates was amplified from scions by immunocapture reverse transcription PCR (IC-RT-PCR) and characterized by RFLP analysis and nucleotide sequencing using strain F13 as reference since it provided the CP transgene. Analysis of EcoRI and StyI RFLP banding patterns from 347 challenging GFLV isolates and sequence data from 85 variants revealed no characteristics similar to strain F13 and no difference in the molecular variability among isolates from 190 transgenic and 157 nontransgenic plants, or from plants within (253 individuals) or outside (94 individuals) the two sites. Interestingly, five GFLV recombinants were isolated in non-transgenic grapevines that were in spatial proximity but outside the two experimental field sites. This survey indicates that transgenic grapevines did not assist the emergence of viable GFLV recombinants to detectable levels nor did they affect the molecular diversity of indigenous GFLV populations during the trial period.

14.5.2.2 Prunus *spp.*

Most cultivated Prunus species are highly susceptible to "sharka" or plum pox disease, caused by Plum pox virus (PPV; for details see Chap. 9). Transgenic plums transformed with the plum pox virus coat protein (PPV CP) gene displayed a resistance to the sharka disease (Ravelonandro et al. 1997). However, the expression of PPV CP in transgenic plants may lead to complementation of deficient characteristics of an incoming potyvirus. Indeed, an aphid-intransmissible strain of zucchini yellow mosaic virus (ZYMV-NAT) could be transmitted when encapsidated by the engineered PPV CP (Lecoq et al. 1993). To control such a risk, new PPV CP constructs were designed and transferred to *Nicotiana benthamiana* genome (Jacquet et al. 1998). In the first construct, the DAG amino acid triplet involved in the potyvirus aphid-transmission was deleted. The second construct encoded a truncated PPV CP lacking its first 140 amino acids. In the last construct, the nucleotides encoding the charged amino-acids R^{220}, Q^{221} and D^{264} localized in the core of the PPV CP were removed. Resistant lines producing high level of wild type or modified PPV CP were then inoculated with ZYMV-NAT to perform an aphid-transmission assay. Results of these experiments demonstrated that the use of modified forms of PPV CP genes in transgenic plants provide a good way to control the biological risks associated with heteroencapsidation (Jacquet et al. 1998).

14.6 Conclusions

Most concerns regarding genome instability in transgenic plants are actually not exclusive to those plants but apply for traditional bred and wild plants as well. Woody plants derived from conventional breeding methods have been freely used for many years without similar concerns from the public opinion (reviewed in Predieri 2001). Those plants have, unlike GM plants, unspecific genome changes which should also be considered as risk factors.

Gene silencing, recombination, mutations, activation of retrotransposons or endogenous viruses, and many other forms of genome instability, are risk factors common to both transgenic and non-transgenic plants.

The huge genome plasticity detected in plants pose the question as to whether it makes sense presenting GM plants as the only group of plants representing a biosafety risk factor regarding genome instability. In fact, there is no evidence to support such a statement.

Although GM plants do not seem to have a more instable genome than other plants, there is a demand for more information supporting their risk free status by some non-governmental organizations opposed to genetic engineering. However, biosafety research will probably never be able to detect all possible genome instabilities in GM plants, just as it will not be possible for wild or traditional bred plants. Risk assessment of GM plants should try to clarify at most if these plants can be more prone to genome instability than non-transgenic wild and traditionally bred plants are.

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15 Investigation of Horizontal Gene Transfer from Transgenic Aspen to Ectomycorrhizal Fungi

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15.1 Introduction

The introduction of molecular methods into plant breeding has offered the possibility to construct genetically modified plants with desired qualities. Major goals of genetic engineering are an enhanced growth rate of plants, improvement of product quality, and increased abiotic and biotic stress resistance.

Forest plantations strongly differ from agriculture in that trees have long lifetimes, ranging from around ten years in "short term" rotation cultures (*Salix* or *Populus*) to a few hundred years for species such as beeches or oaks. Since transgenic organisms always harbor some potential risks, the importance of biosafety in genetically modified (GM) forest tree plantations must be underlined.

Four different aspects of biosafety have to be taken into account (and investigated in field experiments) prior to commercial production (see also Chap. 14).

First, the expression of the new properties of genetically modified plants (GMPs) has to be stable over time. Also genomic rearrangements as consequence of transgene integration have to be rules out.

Second, all plant characteristics (not directly affected by the transgenic properties) should remain unaffected in the GMPs compared to their non-transgenic parental breeds.

Third, negative effects outgoing from GMPs on the environment and particularly on non-target organisms have to be avoided. Especially in the case of pathogen resistant GMPs, an impact on non-target organisms (e.g. like the negative effect of Bt transgenic pollen on larvae e.g. monarch butterfly; Hansen and Obrycki 2000; Losey et al. 1999), or a decrease in plant growth-promoting microbial populations, such as bacteria or ectomycorrhizal fungi (Staehelin et al. 2001) has to be investigated.

Fourth, uncontrolled spreading of transgenic trees as well as their modulated genetic information into natural populations (gene flow) must be

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avoided. Three potential spreading possibilities can be distinguished (Wolfenbarger and Phifer 2000): (a) the establishment and distribution of self-sustaining GMP populations by fertile seeds, (b) an introgression of genes into wild populations by pollination, and (c) a horizontal transfer of genes (HGT) from GMPs towards microorganisms (e.g. bacteria or fungi).

15.2 Horizontal Gene Transfer Between Plants and Microorganisms

Since plant DNA has been shown to remain in soil over extended periods of time (Gebhard and Smalla 1999), concerns that DNA from transgenic plants may spread horizontally to microorganisms have been raised.

Transformation of different bacterial species by using total DNA or tissue material from genetically modified plants has been demonstrated under optimized laboratory conditions (Gebhard and Smalla 1998; Schlüter et al. 1995) as well as in soil microcosms (Nielsen et al. 2000). Since exchange of genetic information between different bacterial species by transformation, transduction or conjugation seems to be common in nature (Krishnapillai 1996; Wöstemeyer et al. 1997), a fast distribution of new genetic information as result of a horizontal gene transfer could be supposed.

However, while transformation of bacteria is common under optimized laboratory conditions, all experiments indicated that the frequency of horizontal gene transfer to bacteria is low under natural conditions. Even under otherwise optimized conditions (e.g. use of purified DNA from transgenic sugar beets as source for the npt*II* gene, and construction of an *Acinetobacter* strain carrying a deleted *npt*II gene to allow homologous recombination in the recipient bacteria), the frequency of HGT was below the detection limit in non-sterilized soil (Nielsen et al. 2000). Also in a field release experiment with *npt*II-transgenic sugar beets, none of the isolated 4000 kanamycin resistant bacterial colonies carried the introduced *npt*II gene (Gebhard and Smalla 1999).

While horizontal gene transfer events between plants and bacteria are relatively well investigated, data involving fungi are less available. Detailed analysis of DNA and amino acid sequence data of genome sequencing projects has indicated that horizontal transmission of genes probably occurred, even if in rare cases, during evolution between eukaryotes from different systematic kingdoms (Dröge et al. 1998). By a combined analysis of G/C content and patterns of codon usage, Garcia-Vallvé et al. (2000) found indications for a transfer of an endoglucanase (*celA*) gene from the rumen bacterium *Fibrobacter succinogenes* to the rumen fungus *Orpinomyces joyoni*. Also a direct gene transfer from dead plant material to the saprophytic fungus *Aspergillus niger* has been reported (under laboratory conditions) (Hoffmann et al. 1994). However, these investigations also indicate, that gene transfer events are rather rare.

Since the availability of free DNA in soil is a limiting factor for horizontal gene transfer (Gebhard and Smalla 1999), and because some fungi grow in an intimate contact with (ectophytic fungi) or even within plants (endophytic fungi), uptake of plant DNA by these fungi might be more likely. Evidence for this comes from the observation that the phytopathogenic fungus *Plasmodiophora brassicae* takes up host plant DNA during each of its infection cycles (Bryngelsson et al. 1988).

In this contribution we want to focus on one particular type of plant fungus-interactions, the ectomycorrhizas (ECM).

15.3 Ectomycorrhizal Fungi and Horizontal Gene Transfer

15.3.1 What Makes Ectomycorrhizal Fungi Interesting with Respect to Horizontal Gene Transfer?

ECM are ectosymbiotic associations of fine roots, mostly of trees, with filamentous soil fungi, mostly of asco- and basidiomycete genera. The dominating tree families of boreal and temperate forests, *Fagaceae* and *Pinaceae*, are ecologically obligate bearer of ectomycorrhizal symbionts. In this symbiosis both root and fungus no longer function independently, but form a unit with adapted metabolic pathways and controlled exchange of metabolites (Smith and Read 1997).

Completely developed ECMs are structurally characterized by the presence of two fungal networks: (1) a fungal sheath that is covering the plant root and provides a barrier against pathogen attacks, and (2) the Hartig net, highly branched hyphae that penetrate between root epidermal and cortical cells and establish a close contact zone with the host root. During ectomycorrhizal symbiosis, root cells degenerate frequently and thus liberate (plant) DNA. Since DNA uptake by filamentous fungi often leads to its integration into the fungal genome, a stable horizontal gene transfer is possible.

Due to its function as exchange "organ", fungal and plant cells of the Hartig net have a highly enlarged plasma membrane surface area (Kottke and Oberwinkler 1987), that might favor DNA-uptake by fungal hyphae. Since trees have long lifetimes, and fine roots of most forest trees are predominantly mycorrhized under natural conditions, horizontal gene transfer might be a more frequent event in ECM symbiosis than in free associations in soil.

15.3.2 Investigation of Horizontal Gene Transfer from Trees to Ectomycorrhizal Fungi under Laboratory Conditions

Up to now, two different approaches were used to study horizontal gene transfer from trees to fungal hyphae in ectomycorrhizas.

In a study of Kaldorf et al. (2004), transgenic aspen, containing the *rolC* gene from *Agrobacterium rhizogenes* under the control of the light-inducible

plant *rbcS* promoter (Fladung et al. 1997), were used. Small aspen cuttings were grown in vitro together with the ectomycorrhizal ascomycete *Phialocephala fortinii*, isolated from mycorrhized roots obtained from an aspen plantation (Fladung et al. 2000). After 12 weeks of co-cultivation, *P. fortinii* mycorrhizas were isolated and transferred to Petri dishes containing fungal growth medium. To avoid contamination with plant material, genomic DNA was only isolated from fungal hyphae that were growing out of mycorrhizas. The quality of the isolated fungal DNA was checked using the ITS1/ITS4 primer pair (White et al. 1990), and the occurrence of horizontal gene transfer was analyzed by the amplification of the fungal DNA with nested *rolC* gene primers. No single *rolC* signal was detected in any of the 24 analyzed *Phialocephala* colonies (Kaldorf et al. 2004). Unfortunately, only a small number of replicates was tested in this study. However, the results indicate that in contrast to what has been observed in *Plasmodiophora brassicae*-plant interaction (Bryngelsson et al. 1988), uptake of plant DNA by fungal hyphae does not occur in *Phialocephala* ectomycorrhizas on a regular basis.

In a second approach (Nehls et al. 2000; Zhang et al., 2005), transgenic aspen, containing the *Streptococcus hygroscopicus bar* gene conferring herbicide (BASTA) resistance under the control of a fungal GPD promoter (van Wert and Yoder 1994) were generated. Proper function of the BASTA resistance construct had been previously tested by the successful transformation of the BASTA-sensitive ectomycorrhizal fungus *Amanita muscaria* (Zhang et al., 2005). Mycorrhizas were formed under axenic conditions between transgenic aspen and wild type hyphae of *A. muscaria* (Nehls et al. 2000) using a Petri dish system (Fig. 15.1) according to Hampp et al. (1996). To detect horizontal gene transfer events, a total of 35,000 ectomycorrhizas (from 1000 transgenic aspen plants representing 15 independent transgenic and control clones) were dissected and tested for BASTA resistance. The utilization of the BASTA resistance marker had the advantage, that a large sample number could be simultaneously screened while only a small number had to be investigated in detail (those that were able to grow under selective conditions).

From the 35,000 investigated mycorrhizas, 102 fungal colonies were formed under selective conditions. However, since these fungal isolates stopped growth when transferred to fresh selection plates, and no *bar* gene could be amplified from fungal DNA by PCR, these fungal colonies were characterized as false positives (Zhang et al., 2005).

The 35,000 mycorrhizas investigated in this study of course only represent a limited sample number. Nevertheless, each mycorrhiza contains a large number (few thousand) of fungal cells in direct contact with plant epidermal cells, that degenerated under the selection conditions. Therefore, the sample size was sufficient to show that, at least under axenic conditions, horizontal gene transfer from the plant root cells to the fungal partner is, if it ever occurs, a rare event in ECM symbiosis.

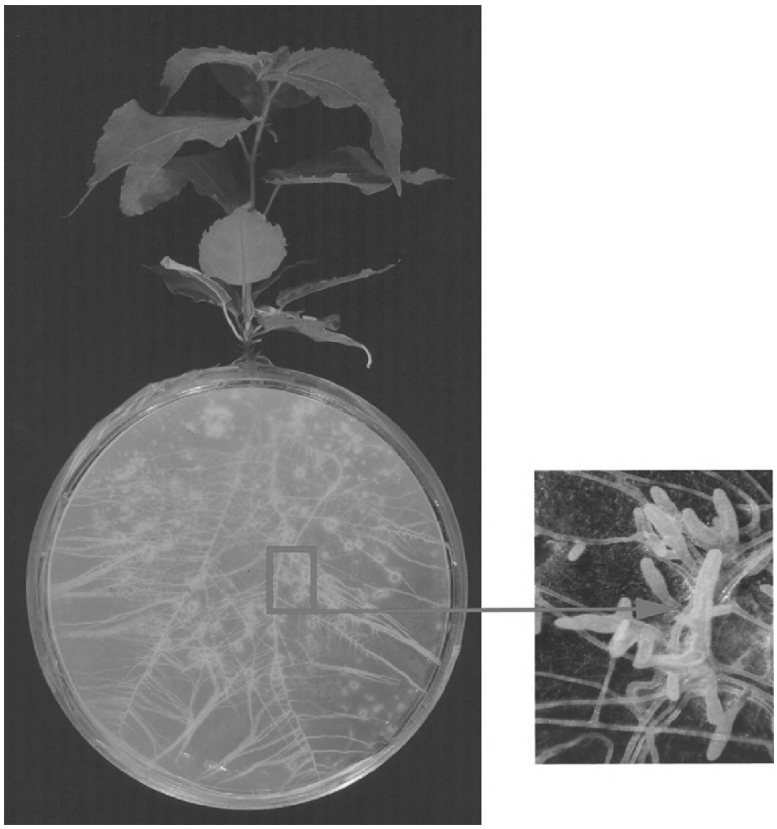

Fig. 15.1. Rooted aspen cuttings were transferred to Petri dishes and the root systems were inoculated with homogenized fungal mycelium according to Hampp et al. (1996). Mycorrhiza formation (see close-up on the right) occurred after six to eight weeks of co-inoculation

15.3.3 Investigation of Horizontal Gene Transfer from Aspen to Ectomycorrhizal Fungi under Field Conditions

In nature, ectomycorrhizas are additionally associated with viruses, bacteria and pathogenic as well as saprophytic fungi, organisms that might significantly influence horizontal gene transfer. Thus, to investigate horizontal gene transfer from aspen to ectomycorrhizal fungi under natural conditions, transgenic trees containing the *bar* gene (conferring BASTA resistance, see Sect. 15.3.2) were released in a field experiment.

15.3.3.1 Experimental Site and Planting Conditions of Aspen

The release experiment was conducted in a closed 1000-m^2 large field station at the Federal Research Center for Forestry and Forest Products

(Grosshansdorf, Germany) near to a plot (20 m distance) where ECM fungal populations of transgenic aspen from a previous liberation experiment were analyzed (for details see Kaldorf et al. 2002). One site of the plot was lined with a small Norway spruce forest and a second with deciduous trees. The loamy soil was homogenized by ploughing and harrowing and covered by a well-mineralized mull prior to the setting out of six-month-old GM aspen grown in the greenhouse.

15.3.3.2 Sampling and Analysis of Ectomycorrhizal Biodiversity

In each sampling season (May and September; 2001 and 2002) the root systems of at least 14 transgenic aspen (between 1.5 and 3 years old), taken from different parts of the plot, were dug up together with the surrounding soil, and stored in plastic bags at 4 °C for up to a maximum of 2 months (after this time the vitality of the ECM fungi declined significantly). For the isolation of mycorrhizas, soil cores were removed carefully from the roots by washing with tap water. Fine roots were detached and rinsed properly under tap water for a minimum of 10 min. Single mycorrhizas (between 1500 and 5000 from each root system) were isolated using a dissection microscope and forceps, and stored in ice water prior to surface sterilization and plating onto selection media (see below).

To evaluate the different ectomycorrhizal fungi that colonized the investigated aspen plants, morphotyping was performed according to Agerer (1987–1993). Color, texture, shape and branching pattern of ECM were used as criteria. On this basis, six different ECM morphotypes were differentiated (Table 15.1).

The determined ECM morphotypes were comparable to those observed in the earlier release experiment of GM aspen just nearby (Kaldorf et al. 2002, 2004) (see Table 15.1 for comparison). The white WL (EM 6.2 in Kaldorf et al. 2002), the white WR (EM 6.1, EM 22 in Kaldorf et al. 2002) and the brown BL

Table 15.1. Comparison of isolated ectomycorrhizal morphotypes (this study) with those obtained in a previous field experiment with transgenic aspen from a plot located near by Kaldorf et al. (2002)

Abbreviation	Color and shape	Description in Kaldorf et al. (2002):
WR	White round and short	*Laccaria* sp. EM 6.1, *Agaricales* EM 22
WL	White long	*Tomentella* sp. EM 6.2
DR	Dark round and short	*Phialocephala* EM 5, *Cenococcum* EM 18
DL	Dark long	*Thelephoraceae* EM 20
BeL	Beige long	*Tomentella* sp. EM 6.2, *Boletaceae* EM 16
BL	Brown long	*Thelephoraceae* EM 14, *Pezizales* EM 2

(EM 14, EM 2 in Kaldorf et al. 2002) ECM morphotypes were most abundant in both studies. However, in contrast to Kaldorf et al. (2002), the dark morphotypes DL and DR (EM5, EM 18, EM 20 in Kaldorf et al. 2002) were less frequent in our investigation.

To confirm the morphotyping data, genomic fragments of rRNA genes were amplified from isolated DNA of single mycorrhizas using the ITS1/ITS4 primer pair (White et al. 1990), and used for direct sequencing. The determination of the ectomycorrhizal fungi was performed by comparison of the DNA-sequences with public DNA databases. Ectomycorrhizal *Hebeloma* strains were frequently amplified from aspen ECM type WL, and fungi from the genus *Peziza* were often detected in brown mycorrhizal roots of the category BL. Kaldorf et al. (2004) detected *Hebeloma* from EM 23 morphotype (white morphotype) and *Peziza* from EM2 and EM10 morphotypes (brown morphotypes) by similar means from aspen mycorrhizas, indicating that the morphotyping of mycorrhizas was comparable in both studies.

Similar patterns of ectomycorrhizal morphotypes in both experiments are probably the result of the following factors: similar soil, similar surrounding with forest trees, same plant genus (*P. tremula* × *tremuloides*) and comparable age of the analyzed root systems (between 1.5 and 6 years). However, even when the overall abundance of ECM morphotypes in both studies was comparable, a strong variation between individual plants and also between seasons was observed (Fig. 15.2; see also Kaldorf et al. 2004). In our investigation, ECM biodiversity was highest in October 2001 and May 2002. We suppose that the reason for the overall differences in ECM biodiversity might be due to differences in soil moisture. Both autumn 2001 and spring 2002 (revealing a high biodiversity of ECM fungi) were wet, while spring 2001 and autumn 2002 (revealing a low biodiversity) were dry, owing to low amounts of rain fall. The observed variations in ECM biodiversity were not due to the transgenic properties of the plants, a result that is similar to the conclusion by Kaldorf et al. (2002). Non-transgenic controls that were harvested together with the transgenic trees revealed comparable patterns of ECM colonization at all times of harvest (data not shown).

15.3.3.3 Investigation of Horizontal Gene Transfer

Isolated mycorrhizas were surface sterilized in 30 vol% hydrogen peroxide for 5–20 s, depending on mantle thickness. Sterilized mycorrhizas were washed three times with sterile water and cut into 2–3 mm long segments. Mycorrhizal segments were than cultivated on 1/2 MMN, or 1.5% potato dextrose agar (only black morphotypes), containing 1–5 µg/ml benomyl and 35 µg/ml Streptomycin sulfate to avoid the growth of bacteria and saprophytic fungi (preferentially ascomycetes) according to Robin Sen (The Macaulay Institute, Environmental Sciences Group, Craigiebuckler, Aberdeen, UK). As soon as fungal colonies started to grow out of the mycorrhizas, small pieces

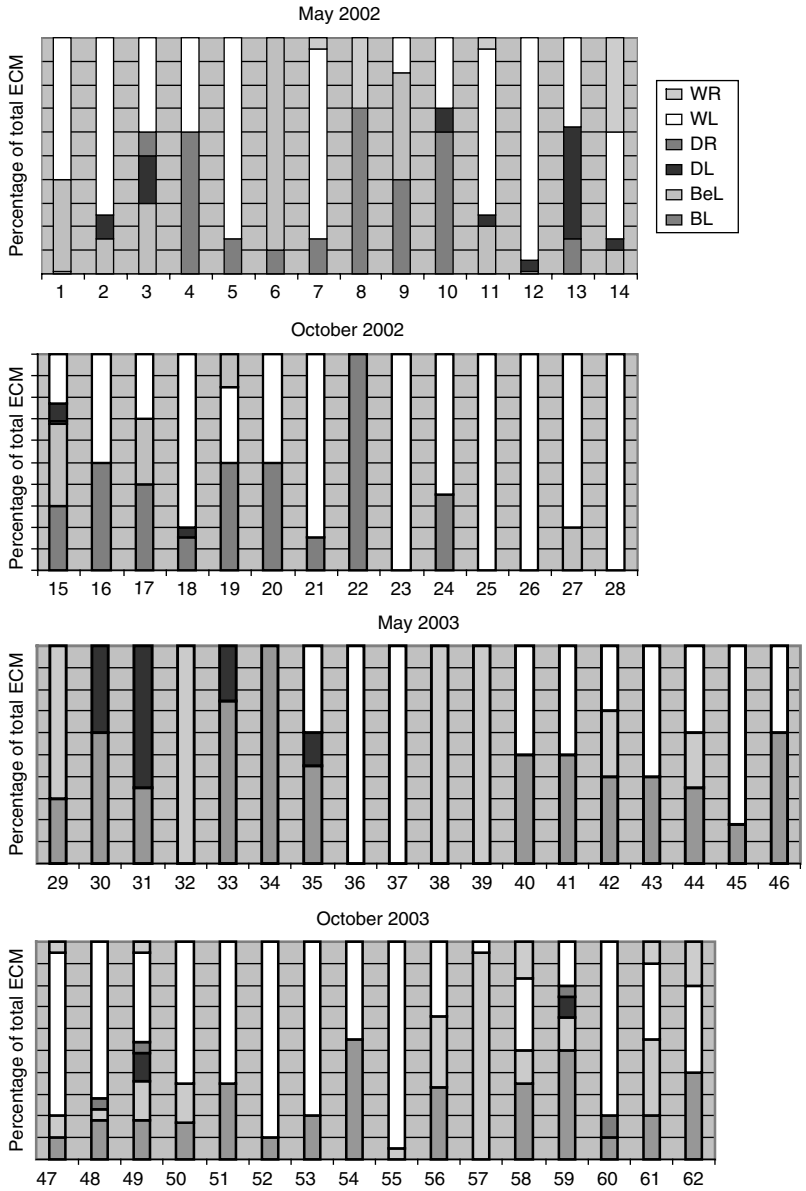

Fig. 15.2. Six different ectomycorrhizal morphotypes could be distinguished in ectomycorrhizas obtained from the field experiment. The portion (in %) of these morphotypes obtained from each of the isolated aspen plants is shown for the four sampling dates. The nomenclature of the morphotypes is identical to the one in Table 15.1: WR = white round, WL = white long, DR = dark round, DL = dark long, WbeL = light beige long, BeL = beige long, BL = brown long, YL = yellow long

of the fungal mycelium were cut off and transferred to selection agar plates (MMN or potato dextrose agar) containing 200 µg/ml BASTA.

From 120,000 investigated mycorrhizas transferred to the selection medium, about 40,000 were revealed to be BASTA-tolerant. These BASTA-tolerant fungal isolates could be divided into two groups: the majority (95%) of these fungi was fast-growing, and colonized a Petri dish within a few days. According to anatomical features (e.g. formation of vegetative spores) and ITS-sequencing of selected morphotypes, these fungal isolates turned out to be saprophytic fungi (mainly *Aspergillus*), that survived surface sterilization and growth suppression by benomyl.

A second, smaller portion (5%) of the BASTA-tolerant fungal isolates were growing much more slowly. According to their growth properties (growth speed) and anatomical features (color, branching pattern) they could be divided into 14 different isolates. The amplification of genomic rRNA fragments revealed, that the isolates were also ascomycetes. However, since they included previously characterized root endophytes, but not ECM fungi, this second class of fungi is presumably representing endophytic fungi, colonizing aspen fine roots in addition to mycorrhizal fungi.

To test whether the BASTA-tolerance of these fungal isolates was a consequence of horizontal gene transfer events, PCR amplification series were initiated to check the presence of the *bar* gene. Genomic DNA was isolated from fungal hyphae and PCR amplification was performed using different *bar* gene specific primer pairs. However, no *bar* gene signal was ever obtained from any of the slow growing BASTA-tolerant isolates, indicating that the fungal isolates were naturally herbicide resistant.

15.4 Conclusions

Compared to saprophytic interactions, a horizontal gene transfer was believed to be more likely in mycorrhizal symbiosis due to special anatomical features of the plant/fungus interface. However, even under optimized conditions (selective advantages of transgenic fungi after a horizontal gene transfer), no indication for a gene transfer from trees to ectomycorrhizal fungi were observed, neither under laboratory nor under field conditions. Thus, in contrast to the phytopathogenic fungus *P. brassicae* that takes up host plant DNA during each infection cycle (Bryngelsson et al. 1988), uptake of plant host DNA that exceed a particular size (necessary for the transfer of information) must be a rather rare event in ectomycorrhizal symbiosis.

One reason for this might be the presence of plant cell walls that are (in contrast to phytopathogenic interactions) not degraded but modulated and filled up with electron-dense material in ectomycorrhizal symbiosis (Kottke and Oberwinkler 1987). Furthermore, as shown for other plants, genomic DNA is presumably degraded during the senescence of root cells in aged

ECM. Together with the plant cell wall, that avoids DNA transfer due to its limited pore size, DNA fragmentation during senescence could prevent the transfer of genetic information from trees to the ectomycorrhizal fungi.

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16 Transgenic Temperate Fruit Tree Rootstocks

SERGEY V DOLGOV[1] AND M-VIOLA HANKE[2]

16.1 Introduction

Temperate fruit trees of the genus *Malus* (apple), *Pyrus* (pear) and *Prunus* (almond, apricot, sweet and sour cherry, peach, and plum) used for commercial fruit production and for backyard growers are usually grafted on clonal (asexually propagated) rootstocks. Rootstocks are used to propagate the fruiting scion onto a rooting system, to gain uniformity and precocity in fruiting portion compared to seedlings, to control tree size (vigor), to adopt the tree to adverse soil conditions (pH, drought, texture, drainage), to tolerate soil pests (nematodes, insects, diseases) and to increase hardiness of the tree to low temperatures. Rootstocks can have a major impact on the profitability of an orchard. The ideal rootstock should induce good tree survival, high annual yields, and acceptable fruit color and fruit size. The influence of the rootstock on the fruiting scion depends on the genotype of the scion. Thus, the knowledge about the suitability of a specific scion-rootstock combination is the key factor for a profitable orchard and guaranties the adaptation of the tree to specific environmental conditions. The worse the soil conditions for the tree the more vigorous should be the rootstock. At present most commercial orchards are of intensive production type where dwarf and superdwarf clonal rootstocks are used.

Identification of problems and prioritizing breeding objectives based on those problems are essential first steps in a rootstock improvement program. For all tree fruits, incorporating resistances to critical diseases and pests will facilitate fruit production in a social environment demanding reduction in pesticide usage. Very large initial seedling populations are required to permit suitably rigorous early screening; the breeding team should anticipate odds of $1{:}10^4$ to $1{:}10^6$ that any given seedling will be commercially successful (Cummins and Aldwinckle 1995). Beside resistance to a very broad spectrum of diseases and pests, general rootstock breeding goals are tree longevity and productivity, propagability of the rootstock, and graft compatibility of the scion-rootstock combination. A summary on traditional breeding of rootstocks for tree fruit crops is given by Cummins and Aldwinckle (1995).

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Tree Transgenesis: Recent Developments

Although almost all rootstock improvement programs now rely on conventional breeding methods, through application of genetic engineering a range of rootstocks has been successfully transformed to introduce specific traits such as disease resistance. Genetic engineering allows one to introduce defined genes into rootstocks established for commercial production, avoiding the transfer of undesirable traits. Main areas for genetic transformation in temperate fruit tree rootstocks are disease and pest resistance and environmental stress tolerance. Biotic resistance is focused on fungal, bacterial, viral, nematode, and insect resistance. Abiotic stress tolerance includes tolerance to various soil and climatic conditions, like winter hardiness, tolerance to high temperatures, drought and salinity. Taking into account the predicted climatic changes over the planet Earth, the importance of biotic and abiotic stress will increase due to the occurrence of pathogens and environmental conditions previously irrelevant for temperate climatic zones. Traditional breeding often fails to improve resistance/tolerance mainly due to the use of a narrow germplasm base in crosses for which resistance to biotic and abiotic stress is low or nonexistent. Resistance to biotic and abiotic factors can frequently be found in various wild species of the genus. Their use in traditional breeding leads to the transfer of undesirable traits and the need for further backcross generation which results in a long time breeding process. Resistance to disease and pests is of high importance taking into account the hazards caused to the environment by pesticides, high costs of treatments per hectare and the ineffectiveness in the case of some pathogens, like bacteria. Besides, genetic engineering in rootstocks is also aimed at alteration of the growth habit and rooting ability.

16.2 Overview of Genetic Transformation in Rootstocks

Fruit trees as well as other woody plants are recalcitrant objects for genetic engineering. This is caused by difficulties both in transformation and selection of transgenic cells, and in regeneration of plants from various kind of tissue. Success of plant transformation depends on several factors: (1) effective system of plant regeneration, (2) effective transformation system of plant cells with high regeneration ability (i.e. to transfer genes), and (3) effective selection system of resulted transgenic tissues and regenerants. The low frequency at which transgenic regenerants can be obtained is mainly due to the lack of an efficient method of regeneration, particularly from mature plant tissue.

16.2.1 *Malus* Rootstocks

There are many studies on apple regeneration; protocols have been described for a large number of apple scion and rootstock cultivars (Korban and Chen 1992; Yao et al. 1999). In apple the most effective and reproducible method for

plant regeneration is through adventitious shoot formation. *In vitro* plant leaves are the ideal source of somatic cells for genetic manipulations. The first report on transformation and regeneration of adventitious shoots from somatic tissue in apple was published in 1989 by James et al. for the apple scion cultivar Greensleeves using *Agrobacterium tumefaciens* mediated transformation into leaf explants (James et al. 1989). Subsequently, transgenic plants were produced using different *Agrobacterium tumefaciens* strains and transformation methods for commercially important scion cultivars, like Delicious (Sriskandarajah et al. 1994), Granny Smith (Trifonova et al. 1994), Royal Gala (Yao et al. 1995; Norelli et al. 1996), Jonagold (DeBondt et al. 1994; 1996), Gala, Golden Delicious and Elstar (Puite and Schaart 1996), Mc Intosh (Bolar et al. 1998) and Pinova (Hanke 2002). Successful transformation and plant regeneration were also reported in apple rootstock cultivars (Table 16.1). Mainly the rootstock M26 was used in apple as this rootstock is a widespread semidwarf rootstock in USA and Europe (Holefors et al. 1998; Welander et al. 1998; Borejsza-Wysocka et al. 1999). The protocol for M26 transformation is well developed in a range of laboratories and this genotype has a high tissue culture ability. Maheswaran et al. reported in 1992 on 100% regeneration frequency in M26 and a number of 13 to 14 shoots per explant (Maheswaran et al. 1992). The dwarf rootstock M9, which is widespread in Europe, was rarely used in transformation studies due to its problems in micropropagation and regeneration (Sedira et al. 2001; Zhu et al. 2001).

The main effort in genetic engineering of apple is aimed at the increase of plant resistance to various pathogens. Fireblight caused by *Erwinia amylovora* and crown or collar rot caused by *Phytophthora cactorum* are the crucial rootstock diseases. M9 and M26, the most widespread dwarf and semidwarf rootstocks, are quite sensitive to fireblight, as well as Ottawa 3 and York 9. M26 is rather sensitive to collar rot. Among the first efforts on transferring genes coding for antimicrobial proteins to apple was research at Cornell University, Geneva, USA. In 1994 transgenic plants of rootstock M7 were obtained carrying the attacin E gene which were more resistant to fireblight under greenhouse conditions than the non-transformed cultivar (Norelli et al. 1994). Two-year field tests have confirmed that one line is much more resistant against fireblight as compared to the control (Momol et al. 1996). Later on some other antimicrobial genes of insects (cecropins SB-37 and Shiva-1), the lysozyme gene of hen eggwhite and of the bacteriophage T4 have been transferred to M26 rootstock (Norelli et al. 1994; Aldwinckle et al. 2003).

Application of plant defensin genes (PD) is another way to increase plant resistance against pathogenic microorganisms (Broekaert et al. 1995). In contrast to non-phytogenic antimicrobial proteins (cecropins, magainins, tachyplesins and others), defensins undoubtedly are optimal for expression in plants by virtue of both gene organization and presence of leader sequences, and codon usage. Transfer of defensin genes from black radish and onion into the apple variety Jonagold allowed a product accumulation of up to 0.1–0.8% of total soluble proteins and accordingly an 8- to 32-fold increase of

Table 16.1. Genetic transformation of fruit crop rootstocks

Trait	Genotype	Strain:vector	Genes	Selective agent	Reference
Marker genes	M26	LBA4404:pBI121 CZ707, EHA101:pCGP257	nptII+gus	Km	Maheswaran et al. (1992)
	M26	C58sZ707, NT1 pBI121, p35SGUSint	nptII+ gus-int	Km, Par, Neo, G418	Norelli and Aldwinckle (1993)
	57-545	CBE21:pBI121 EHA105:p35SGUSint	nptII+gus hpt+gus-int	Km Hyg	Dolgov et al. (2000)
	GP 217	CBE21:pBI121 EHA105:p35SGUSint	nptII+gus hpt+gus-int	Km Hyg	Lebedev and Dolgov (2000)
	M26	EHA101	pmi+gus+ gai	Mannose	Zhu et al. (2004)
Disease resistance	M7	LBA4404:pLDB15	nptII+ gus+attE cecropins, lysozyme+ nptII	Par, Km	Norelli et al. (1994)
	57-545	CBE21:pPCV002rs	nptII+rs-afp2	Km	Dolgov et al. (1999)
	M26	EHA105:4 vectors	nptII+attE	Par, Km	Ko et al. (2000)
	AU56-83	LBA4404, EHA105 KYRT1:pBinAR	nptII+ dpo	Km	Hanke et al. (2003)
Habitus modifications	M26	*A. rhizogenes* A4	–	–	Lambert and Tepfer (1992)
	M26	GV3101:pMRK10	nptII+ rolA	Km	Holefors et al. (1998)
	M26	C58C1:pCMB-B:GUS	nptII+gus+ rolB	Km	Welander et al. (1998)
	Colt	*A. rhizogenes* NCPPB 1855	–	–	Gutièrrez-Pesce et al. (1998)
	M26	C58C1:pROKB	nptII+phyB	Km	Holefors et al. (2000)
	M.9/29	C58C1:pCMB-B:GUS	nptII+gus+rolB	Km	Zhu et al. (2001)

	Jork 9	EHA101A pSCV1.6 C58C1:pCMB-B:GUS	nptII+gus-int nptII+ gus+rolB	Km	Sedira et al. (2001)
	Jork 9	*A. rhizogenes* 4 wild strains	–	–	Pawlicki-Jullian et al. (2002)
	BP10030	C58C1 pCMB-B:GUS	npt II+ gus+rolB	Km	Zhu et al. (2003)
Herbicide resistance	GP 217	CBE21:pBIBar	nptII+bar	Km, PPT	Lebedev et al. (2002)
	57-545	CBE21:pBIBar	nptII+bar	Km, PPT	Dolgov and Skryabin (2004)

plant extract toxicity to *Fusarium culmorum* spores (DeBondt et al. 1998). Unfortunately, greenhouse tests carried out on these transgenic apple plants revealed no essential increase in their resistance to the main pathogens of apple, first of all to scab and bacterial burn (Broothaerts, personal communication). In experiments which were carried out at the Institute of Bioorganic Chemistry in Pushchino, Moscow the PD genes cloned from *R. sativus* were transferred to an apple rootstock for resistance to microbial attacks resulting in 26 lines. All lines obtained were successfully proliferated and rooted on medium containing kanamycin and amplified a fragment of the neomycin phosphotransferase II (*nptII)* gene. Transformation frequency varied from 3.3 to 14.7%. Amplification of a 473 bp fragment corresponding PD was observed for 23 out of 26 transformed lines. Western blot analysis of extracts from greenhouse grown apple rootstock plants showed PD gene expression in 14 lines from 20 lines being tested. Three lines failed to show defensin expression in spite of the presence of PD gene in their genomes. Unfortunately, greenhouse and field tests of plants obtained showed no increase in resistance to pathogens. It might be explained by some defects of gene constructs used for transformation (unpublished data).

The attainment of high and stable expression rate of recombinant protein is insufficient for increased pathogen resistance. The endocellular localization of proteins is also very important as both the level and activity of protein during plant development. It was shown by Ko et al. (2000) on apple variety Galaxy and rootstock M26 carrying the attacin E gene fused to a leader sequence providing an apoplast localization of the resulted protein. Such plants accumulated proteins in intercellular space and showed a greater degree of resistance to fireblight infection due to a direct effect on the fireblight bacterium traveling through intercellular spaces.

Recently, keen interest has developed in tissue-specific and pathogen inducible plant promoters. The majority of economic-valuable genes demands tissue-specific expression. Besides, high levels of constitutive expression of some foreign genes, for example in the case of the endochitinase gene (Bolar et al. 2000), has negative influence on viability of transformants. Inoculation of apple rootstocks by *Erwinia amylovora* carrying the *uidA* gene driven by the promoter of the potato pathogen-related gene *prp1*, has raised the level of expression up to 20% compared to plants with a 35S-*uidA* gene (Reynoird et al. 2000).

A major objective of genetic engineering in apple is the improvement of the rootstock rooting ability and root quality. Dwarf rootstocks usually have a less developed rooting system and demand support of the tree which raises the costs of orchard production. For this purpose the rootstock M26 was transformed by a wild strain of *Agrobacterium rhizogenes* A4 (Lambert and Tepfer 1992). In this study only one transgenic clone was obtained which showed an advantage in rooting compared to the control. This clone also contained other T-DNA backbone genes which caused undesirable modifications of the scion, synthesis of opines and others. Similar results were observed in

transformations of the sweet cherry rootstock Colt by *A. rhizogenes* wild strain that contained the plasmid pRi 1855 (Gutierrez-Pesce et al. 1998). For this reason further transformation of apple and pear rootstocks was carried out by individual *rol* genes driven both by own, and virus regulatory sequences. Transformation of the apple rootstock M26 by *rolA* gene resulted in a more dwarf phenotype and better rooting ability using *rolB* gene. In both cases *rol* genes had intact regulatory sequences, and expression of *uidA* gene driven by the *rolB* gene native promoter has revealed tissue-specific gene localization, especially in root meristematic zones (Holefors et al. 1998; Welander et al. 1998). The authors even observed violent rooting of rootstocks M9/29 and York 9 after transformation by the same gene constructs (Sedira et al. 2001; Zhu et al. 2001). Transformation of the poor rooting pear rootstock BP10030 by the same construct has increased rooting efficiency from 5 to 70–100% (Zhu et al. 2003). However, trees of the apple cultivar Gravenstein which were grafted upon transformed by *rolA* and *rolB* genes M26 rootstock did not reveal any significant differences compared to the control (Zhu and Welander 1999). It is necessary to note that applying *rolB* gene driven by its own promoter resulted in intensification of rooting, but very often the presence of more than two to three target gene copies led to a decrease in the positive effect (Sedira et al. 2001). In other experiments, the use of a gene construct with the *rolB* gene driven by the 35S promoter led to reduction of viability and death of transgenic callus (Dolgov and Firsov, unpublished).

In the focus of genetic engineering was also the growth habit of the tree. Thus, reduction of stem length has been achieved in 9 of 13 transgenic M26 rootstock lines expressing a phytochrome B (*phyB*) gene from *Arabidopsis* (Holefors et al. 2000).

Transferring genes of herbicides resistance into fruit trees can appear especially useful for high-quality planting material production, as the latter is influenced by a lot of factors and the presence of weeds in particular. In nurseries weed control meets difficulties, as up-to-date highly effective herbicides with respective ecological characteristics are non-selective, and at a young age fruit plants are more than usually sensitive to herbicides. Other methods are too laborious and are not of sufficient effectiveness. For example, manual weeding in nurseries requires up to 30% of the total input. Chemical methods of plant protection are more effective in intensive orchards, as they avoid mechanical damage of roots, improve water and nutrition regulation of trees, prevent soil erosion, and save human labor. Besides, wide use of a vegetation free zone in the tree row by herbicide treatment and establishment of a perennial grass sod in the rows in temperate climate horticulture makes stocks resistant to the herbicides; hence they become ideally appropriate for such a system of soil maintenance in orchards. Among fruit crops the apple cultivar Royal Gala is the only one which is known to be resistant to treatment by the herbicide Glean under excessive draughts (Yao et al. 1995). In order to obtain herbicide resistant apple and pear rootstocks at the Institute of Bioorganic

Chemistry in Pushchino, Moscow, the most effective strategy, viz. herbicide detoxification, was applied. Unlike others (overexpression or peptid-target alteration) it does not result in accumulation of herbicide in plants. For this purpose several transformations of apple rootstock 57–545 and pear rootstock GP 217 were accomplished by *bar* gene so as to integrate resistance against phosphinothricin (PPT) (an active agent of some herbicides). In total, one clone of the apple rootstock and 17 clones of the pear rootstocks with PPT resistance were obtained. Non-transgenic control plants died on medium supplemented with 2 mg/l PPT, whereas the transgenic apple plants formed roots on medium supplemented with 10 mg/l PPT, and transgenic plants of a pear proliferated without damage on medium with PPT concentration to the point of 300 mg/l and rooted on 15 mg/l (Fig. 16.1). Transgenic plants were adapted to growth in soil and did not demonstrate any aberration from the original clone. The estimation of plant resistance was carried out in greenhouse and in field. Greenhouse plants were treated by Basta herbicide using application rates of 3, 6, 12 and 24 l/hectare (the standard field application is 3–5 l/hectare). All concentrations caused the destruction of the control plants whereas the inflicted damages of the most resistant clones were insignificant even at the highest concentration. Field plants were treated by 20 l/hectares of herbicide, and some clones proved to be resistant to such a high rate.

16.2.2 *Pyrus* and *Prunus* Rootstocks

The first experiments on adventitious organogenesis of pear rootstocks aimed at transformation were carried out using quince A (*Cydonia oblonga*) (Dolcetsanjuan et al. 1991). Later on, investigations were carried out with various pear cultivars (*P. communis*) (Mourgues et al. 1996; Chevreau et al. 1997, 1999) and a wild pear (*P. communis* var. *pyraster* L.) (Caboni et al. 1999). In pear, the main aim of genetic engineering is to increase resistance to fireblight (Malnoy et al. 2000, 2003a,b, 2005; Reynoird et al. 1999), to increase rooting ability and to induce dwarfness (Bell et al. 1999; Zhu and Welander 2000; Zhu et al. 2003).

As early as 1984 (Jones et al. 1984), there was a report concerning successful adventitious regeneration of the cherry rootstock Colt, followed by reports on other rootstocks for sweet and sour cherry (Gutierrez-Pesce et al. 1998; Fang et al. 1999).

16.2.3 Factors Affecting the Transformation Efficiency

Several factors are influencing the transformation and regeneration efficiency in fruit trees. The major factors are: plant genotype used for transformation; *Agrobacterium* strain and the plasmid used; medium composition during selection and regeneration; selective agent used to obtain transformants.

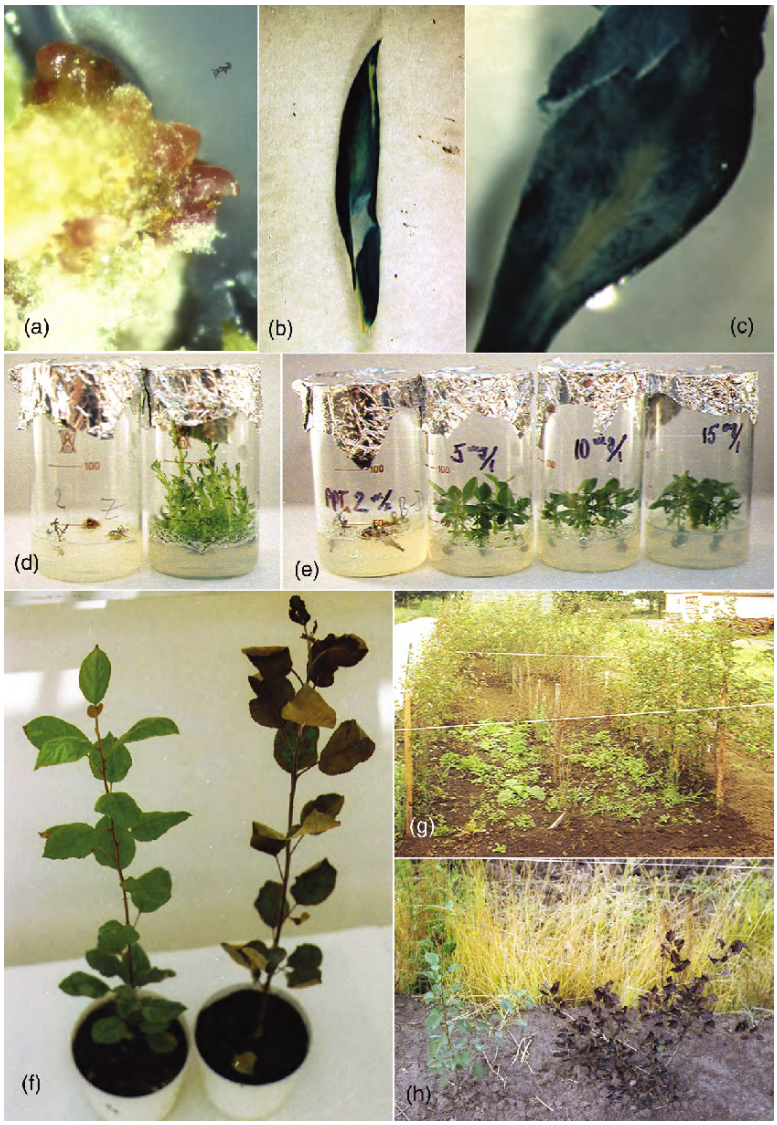

Fig. 16.1(a–h). Adventitious shoot regeneration from callus of apple rootstock: (a) GUS activity in leaf of transgenic pear rootstock; (b,c) *uidA* gene with (b) and without (c) intron; (d,e) multiplication (d) and rooting (e) of pear rootstock transformed by *bar* gene on selective medium (*left* control plants); (f) transgenic apple rootstock after herbicide treatment in greenhouse (*right* control); (g) herbicide treatment of transgenic apple (*middle* control); (h) pear (*right* control) rootstock in the field

The influence of different *Agrobacterium* strains on transformation efficiency is insufficiently known until now. Martin et al. (1990) studied the frequency of callus formation and expression of GUS in callus tissue when apple tissue was

infected by various *Agrobacterium* strains carrying pBI121 as a vector. They reported on a low virulence of the conventional Ach5, LBA4404 strains and a clear advantage of the supervirulent A281 strain. Cocultivation of M26 rootstock leaf explants with three wild strains of *Agrobacterium* carried out by Maheswaran et al. (1992) revealed superiority of A281 among the others as well. As compared to pear, a sufficient tolerance of apple to infection by *Agrobacterium* was shown. A successful transformation in pear demands the use of supervirulent strains (Jones et al. 1984; Dolgov et al. 2000).

The proper choice of the selective agent is essentially important for effective plant transformation. Thus, even if the *nptII* gene was used as the standard selective marker, transformation frequency of several crops was sharply increased during selection on genetycin (G418) (Dekeyser et al. 1988; Howe et al. 1994), but it was absolutely unsuitable for transformation of apple (Norelli and Aldwinckle 1993). It has been shown that paromomycin and neomycin are better for selection of transgenic shoots in apple than kanamycin or genetycin. A few vain attempts were made to use herbicide resistance genes as selective markers for fruit tree transformation. Both DeBondt et al. (1996) trying to apply the *bar* gene which provides phosphinothricin resistance and Yao et al. (1995) using the *als* gene in apple via direct selection on chlorsulfuron failed to get transgenic plants.

16.3 Methodology of Rootstock Transformation and Results Obtained

Material. The semidwarf apple rootstock 57–545 and the pear rootstock GP217 were used in experiments at the Institute of Bioorganic Chemistry in Pushchino, Moscow. The *uidA* gene as a marker (with and without intron), the plant defensine *rs-afp2* gene and the *bar* herbicide resistant gene have been used for transformation experiments.

Transformation. Leaf pieces of about 1 cm^2 (apple) or the youngest 2–3 fully expanded leaves (pear) from *in vitro* rooted plants were used for transformation. The explants were immersed in a bacterial suspension for 40–50 min and blotted on a sterile filter paper, transferred with their adaxial side in contact with a filter paper on culture medium and co-cultivated over 2–3 days at 23 °C in the dark. The cocultivation medium for apple explants contained Murashige and Skoog (1962) salts, 3.0 mg/l 4-chlorphenylurea (4CPU), 1.0 mg/l naphthalene acetic acid (NAA), 1.0 mg/l 2,3,5-tri-iodobenzoic acid (TIBA). The medium for pear explants contained the Nitsch's macro- and microelements (Nitsch and Nitsch 1969), 3.0 mg/l thidiazuron (TDZ), 0.5 mg/l NAA, 0.1 mg/l gibberellic acid 3 (GA_3). For selection 25–35 mg/l kanamycin (Km) or 5 mg/l hygromycin (Hyg) or 5 mg/l PPT and 500 mg/l cefotaxime were added. Every four weeks calluses were replaced to fresh media. Resulted regenerants were propagated on MS media

supplemented with 1–2 mg/l benzylaminopurine (BAP) and successfully rooted on one-half MS media with 0.5 mg/l indolyl butyric acid (IBA); 35 and 10 mg/l Km, or 15 and 5 mg/l Hyg, or 5–3 mg/l phosphinothricin (PPT) were used for these steps accordingly.

Results. An efficient protocol of adventitious shoots regeneration for the apple rootstock 57–545 and the pear rootstock GP217 was developed (Fig. 16.1). Twenty independent transgenic lines of the clonal rootstock 57–545 were obtained in three transformation experiments using the vector pBI121 in the disarmed supervirulent strain CBE21. Transformation frequencies varied from 1.5 to 7.2%. Transformation of this genotype using EHA105 strain and hygromycin as a selective agent allowed one to improve slightly the transformation rate to 8.3%. Nine Kanamycin and 11 hygromycin resistant pear rootstocks have resulted from the *uidA* gene transformation. As in the case of apple, hygromycin has been found to be more effective than kanamycin. Transformation frequency was increased to 6.2–11.5% using hygromycin compared to 0.4–3.1% using kanamycin. The amount of escapes decreased noticeably from 44.0 to 8.3%. Phosphinothricin selection of the pear rootstock explants has led to unusually high regeneration frequency – up to 38%, in comparison with kanamycin – 0.6–5% or hygromycin – 9%. But the vast majority of sprouts (96% on average) have turned out to be non-transgenic. The similar picture was observed for apple as well. The studies showed that PPT stimulated regeneration of apple and pear explants but only after cocultivation with *Agrobacterium*. A similar stimulating effect of PPT on shoot regeneration has already been mentioned in other studies (Hebert-Soule et al. 1995; Hoshino and Mii 1998). Other reports showed that selection of apple on PPT has not given results at all (DeBondt et al. 1996). We obtained only one transgenic clone of the apple rootstock in such a way. While the transformation frequency using kanamycin selection varied from 3.3% to 14.7% in our previous experiments with the same rootstock, the frequency on PPT selection was only 1.3%.

In pear, leafstalks have a number of advantages as explants compared to leaves, such as a higher number of putative transgenic shoots per explant and less agrobacterial contamination. Using the *bar* gene, transformation frequency of leaves was 0.9% in one experiment and futile in two others, while the same rate for leafstalks was 1.0, 2.9 and 2.5%, accordingly.

In 1987 it was noted first that the plant genes with introns have higher expression than without them (Callis et al. 1987). GUS is a very convenient reporter for studies on various factors that affect expression level of transgenes. The GUS gene expression is easy to detect both qualitatively (histochemical method) and quantitatively (fluorometric method). Various constructions of a *uidA* gene were transferred both into apple (Maheswaran et al. 1992; Norelli et al. 1994), and pear rootstocks (Kaneyoshi et al. 2001). The *uidA* gene (with and without intron) was used for transformation of several apple cultivars (Puite and Schaart 1996), but there was no comparison of expression levels. At the Institute of Bioorganic Chemistry in Pushchino,

Moscow, transgenic apple and pear plants carrying two different *uidA* genes (with and without an intron) were obtained. The value of quantitative expression in plants was measured by fluorometric method using greenhouse plants during two (apple) and six (pear) seasons. Differences were detected among clones transformed by the same vector and also between different reported gene constructions. Detectable GUS activity in transgenic apple rootstocks varied from 43 (level of non-transformed control) to 5630 nmoles 4MU/min/mg fresh weight of leaf tissue. These differences remained during two vegetation seasons. A more effective gene expression was observed in one apple rootstock clone transformed by gene *uidA* containing a plant intron (8830 nmoles) (Dolgov et al. 2000). In pear, despite some fluctuations in enzyme activity over time, the presence of an intron amplified activity was similar throughout (tree-fold on average). Plant field tests demonstrated approximately double-increased activity. These results stated a stability and intron-dependent strengthening of transgene expression in pear rootstocks both in greenhouse and in field within years. It was reported previously (Yao et al. 1995) that, over four seasons, GUS activity was observed in transgenic apple leaf tissue and in flowers and fruits as well, but the quantitative test on expression level wasn't carried out. Besides, the stable expression of the GUS-intron transgene in citrus rootstock plants over a period of four to five years was demonstrated (Cervera et al. 2000).

16.4 Field Tests of Transgenic Rootstocks

Field tests of transgenic rootstocks were carried out only in a few countries. Apple rootstocks transformed with antimicrobial genes (attacin, cecropin and lysozyme) were released for field trials since 1993 at Cornell University, USA. Field tests on plants with chitinase genes started in 1998–1999 at the same place. In 1998 field tests of the cherry rootstock Colt carrying the whole T-DNA of *A. rhizogenes* were carried out in Italy (Rugini and Gutierrez-Pesce 1999). Since 1999, tests of transgenic apple rootstocks (M26 and M9) have been put into practice in Sweden. In 2004 pear rootstock BP10030 was added. The *rolB* gene has been transferred into these rootstocks which were used for five grafted apple and three pear varieties. In Russia, field tests of clonal apple and pear rootstocks transformed by marker, plant defensin and herbicide resistance genes have been carried out since 2000.

16.5 Conclusions

In temperate fruit trees, efficient transformation systems have been developed in several laboratories for scion as for rootstock cultivars. The main research has been carried out in *Malus* due to the economical importance of this crop

worldwide. In *Prunus*, research is focused more on disease resistance of scion cultivars, for instance in plum. Although some investigations on rootstocks are promising, only a few research groups are concentrating on rootstocks as breeding activities in this field are rare. It is beyond any doubt that genetic engineering will become a powerful tool in breeding, especially in fruit trees. The high degree of heterozygosity, the extensive reproduction cycle with a long juvenile period, and the complex reproductive biology based on self-incompatibility are problems in conventional breeding of tree species. Gene transfer for fruit tree improvement has several advantages to overcome these problems. Recently, Petri and Burgos (2005) reported on advantages and future perspectives in fruit tree transformation. They stated that the future of genetic transformation as a breeding tool requires the development of genotype-independent procedures. Besides, the increase of knowledge in genomics, the identification and isolation of plant genes which naturally occur in fruit trees, will contribute to the application and public acceptance of this tool in breeding.

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