Trees (2002) 16:250–261
DOI 10.1007/s00468-002-0178-2

REVIEW

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Transgenic trees as tools in tree and plant physiology

Received: 8 February 2002 / Accepted: 8 February 2002 / Published online: 28 March 2002

Abstract Transgenic trees are major products of tree biotechnology. This relatively young field of both plant biotechnology and tree biology concentrates on (1) improvement of pathogen, pesticide, and stress resistance, (2) manipulation of lignin content and composition, and (3) improvement of growth. Transgenic trees also have a great potential in other areas of applied and environmental research, such as in the production of phytochemicals and in phytoremediation of polluted soils. However, genetically modified trees are also excellent tools for physiological research. Transgenic trees are indispensable in investigations of the regulation of wood formation, long-distance transport, and tree growth cycles. In addition, transgenic poplars contribute significantly to our understanding of the regulation of sulfur nutrition. In this review we concentrate on the use of transgenic tree species to improve knowledge in tree and, more generally, plant physiology rather than to cover extensively the field of commercial tree biotechnology or the biological safety of transgenic plant release.

Keywords Transgenic trees · Sulfur metabolism · Stress physiology · Hormones · Reproduction biology

Introduction

Since the first successful transformation of a tree species in 1987 (Fillatti et al. 1987) transgenic trees have become essential tools for forest tree biotechnology and tree physiology. Trees have proved to be as suitable as herbaceous plants as subjects for molecular methods of basic research and tree breeding. This was demonstrated, for example, by construction of expressed sequence tag (EST) libraries from pine and poplar xylem tissues (Allona et al. 1998; Sterky et al. 1998), successful application of promoter analysis with *gus* or green fluorescent protein reporter genes (Tian et al. 1999; Chen et al. 2000), optimization of in situ RNA hybridization in poplar xylem tissue (Regan et al. 1999), and the use of proteomics to address the protein composition of xylem (Van der Mijnsbrugge et al. 2000). Despite the drawbacks of long breeding cycles and large genome sizes, biotechnological approaches have and will have a great impact on forestry and tree breeding. Genetic maps based on random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), or microsatellite markers have been constructed for several tree species including poplar, *Eucalyptus*, oak, *Acacia*, Douglas-fir, and pine (Krutovskii et al. 1998; Cervera et al. 2000; Moran et al. 2000). These markers were utilized to map quantitative trait loci (QTL), e.g. for growth parameters in pine and poplar (Wu 1998; Kaya et al. 1999), timing of bud set and flush in poplar (Frewen et al. 2000), or wood properties in loblolly pine (Sewell et al. 2000).

Many transgenic trees were generated to address specific questions in tree physiology and tree biotechnology (Fig. 1). The field of forest biotechnology has been subjected to several recent reviews (Séguin et al. 1998; Robinson 1999; Merkle and Dean 2000; Dinus et al. 2001; Peña and Séguin 2001; Van Raemdonck et al. 2001). This review aims to overview the use of transgenic trees to improve knowledge in tree and, more generally, plant physiology, but not to cover in detail commercial tree biotechnology and lignin manipulation (Baucher et al. 1998; Whetten et al. 1998; Grima-Pettenati and Goffner 1999; Sederoff et al. 1999), or to discuss the preferences and disadvantages of transgenic trees for forest management (reviewed by Jouanin 2000) or the problems connected with the release of genetically manipulated forest trees into the natural environment (reviewed by Mullin and Bertrand 1998).

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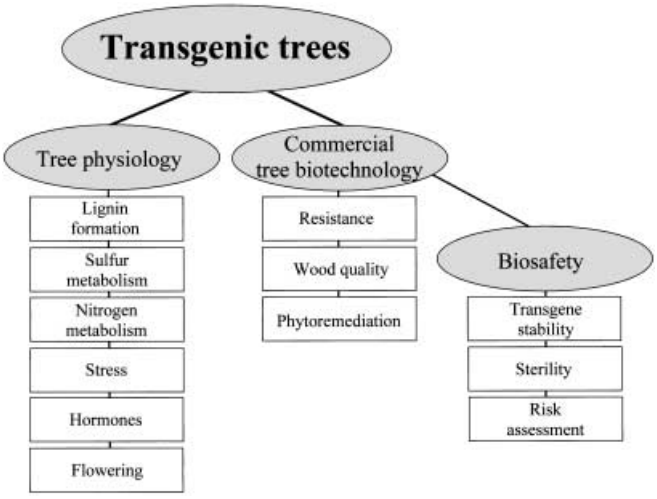

Fig. 1 Transgenic trees in tree physiology and biotechnology

Commercial tree biotechnology

The number of tree species for which in vitro propagation and transformation protocols exist is growing (reviewed in Tzfira et al. 1998a; Bajaj 1999), as well as the number of genetically modified trees being tested in field trials (approx. 140 field-trials with 17 species) (Mullin and Bertrand 1998; McLean and Charest 2000). Beside the pioneer genus *Populus* tree biotechnology concentrates on transformation of conifers, where only a few stable transgenic lines exist, and economically important species such as *Eucalyptus*, grape, and apple.

As in crop plant biotechnology, the first traits expressed in trees and tested in field release experiments were insect, herbicide, and disease resistance (Jouanin et al. 1998; Séguin et al. 1998; Robinson 1999). Genes coding for *Bacillus thuringiensis* toxins were introduced into several tree species including poplar, walnut, white spruce, and larch (reviewed in Jouanin 2000). Another strategy for engineering insect resistance was employed by overexpressing proteinase inhibitors in poplar, resulting in toxicity of such plants for *Chrysomela tremulae* (Leple et al. 1995) or *Plagiodera versicolora* (Klopfenstein et al. 1997). Transgenic aspens and poplars were manipulated for resistance against the herbicides glyphosate (Donahue et al. 1994; Strauss et al. 1997), chlorsulfuron (Brasileiro et al. 1992), and phosphinotricine (DeBlock 1990; Jouanin et al. 1993). Papaya overexpressing the ring-spot virus coat protein and thus less susceptible to infection by this virus (Gonsalves 1998) was the first commercial transgenic tree (McLean and Charest 2000; Chiang et al. 2001).

Lignin is a complex phenolic polymer which is located in the cell wall of higher plants and has essential functions for mechanical support, solute transport, and disease resistance. However, lignin must be removed from wood during paper manufacture, which is connected with considerable energy demand, chemical consumption, and environmental pollution. Therefore, reduction of lignin content and manipulation of its composition are among the most important targets of tree biotechnology, as documented by the number of recent reviews on lignin biosynthesis and accomplishments of genetic engineering in the manipulation of lignin (Campbell and Sederoff 1996; Douglas 1996; Baucher et al. 1998; Whetten et al. 1998; Grima-Pettenati and Goffner 1999; Sederoff et al. 1999; Merkle and Dean 2000; Peña and Séguin 2001; Van Raemdonck et al. 2001).

Although the major traits introduced into tree species, i.e. insect resistance, low lignin, enhanced growth, or potential for phytoremediation, promise a positive impact on the environment and, in the long term, a preservation of natural forests, there are several possible risks to the native forest ecosystems (reviewed in Mullin and Bertrand 1998; Mathews and Campbell 2000). Increasing knowledge about the control of flower development in trees, however, opens up strategies to reduce or prevent the danger of vertical gene transfer to the wild tree species via genetic engineering of sterility (Strauss et al. 1995). Another problem specific to tree species, compared to the conventional agriculture crops, is the necessity for long-term stability of the transgene over several vegetation periods (Fladung 1999; Kumar and Fladung 2001). In addition, risk assessment investigations aimed at, for example, root-soil interactions, transfer of the transgene to the wild plants, and possibility of horizontal gene transfer must be part of field-trials with transgenic trees as discussed by McLean and Charest (2000), Peña and Séguin (2001), or Strauss et al. (2001).

Tree physiology

Transgenic trees in sulfur metabolism

Sulfur is an essential element found in plants mostly in its reduced form in amino acids cysteine (Cys) and methionine (Met). Plants take up sulfur in the oxidized form of sulfate, reduce it, and incorporate it into the amino acid skeleton of *O*-acetylserine forming Cys. Cys can be utilized for synthesis of proteins or be further metabolized to Met, glutathione (GSH) (Leustek et al. 2000; Saito 2000), secondary sulfur-containing compounds (Schnug 1993), and phytochelatins (Rauser 1999; Cobbett 2000). The tripeptide GSH fulfils various important metabolic and regulatory functions such as (1) sulfur storage, transport, and regulation of sulfate uptake and transport (Herschbach and Rennenberg 2001a), (2) protection against oxidative stress caused by active oxygen species (AOS) (Polle and Rennenberg 1994; Noctor and Foyer 1998), (3) detoxification of xenobiotics after conjugation by glutathione-S-transferase (GST) (Lamoureux and Rusness 1993), (4) detoxification of heavy metals via phytochelatins (Rauser 1999; Cobbett 2000), (5) building and maintenance of protein tertiary structure and reactive states (Gilbert 1990; Kunert and Foyer 1993), and (6) regulation of gene expression (Wingate et

Table 1 List of transgenic trees overexpressing genes involved in sulfur or nitrogen metabolism

Tree species	Gene overexpressed	Origin of the coding sequence	Compartment	Promoter	Line coding	Literature
Populus tremula × *P. alba*	γ-Glutamylcysteine synthetase	*Escherichia coli*	Cytosol	CaMV 35S	gsh28 ggs	Noctor et al. (1996); Arisi et al. (1997)
Populus tremula × *P. alba*	γ-Glutamylcysteine synthetase	*Escherichia coli*	Chloroplast	CaMV 35S	Lggs	Noctor et al. (1998a)
Populus tremula × *P. alba*	Glutathione synthetase	*Escherichia coli*	Cytosol	CaMV 35S	gsh	Foyer et al. (1995); Arisi et al. (1997)
Populus tremula × *P. alba*	Glutathione synthetase	*Escherichia coli*	Chloroplast	CaMV 35S	Lgsh	Noctor et al. (1998a)
Populus tremula × *P. alba*	Glutathione reductase	*Escherichia coli*	Cytosol	CaMV 35S	35 gor	Foyer et al. (1995)
Populus tremula × *P. alba*	Glutathione reductase	*Escherichia coli*	Chloroplast	CaMV 35S	70 L gor	Foyer et al. (1995)
Populus tremula × *P. alba*	Glutamine synthetase	*Pinus pinaster*	Cytosol	CaMV 35S		Gallardo et al. (1999)

al. 1988; Link et al. 1997). To investigate the mechanisms of GSH action in these processes several plant species were manipulated in GSH synthesis. Transgenic poplars overexpressing bacterial genes for enzymes involved in GSH synthesis represent the best characterized species and contributed significantly to our knowledge of regulation of sulfur nutrition and GSH synthesis in plants (Table 1).

GSH is synthesized in two steps. First, Cys is joined to glutamate in reaction catalysed by γ-glutamylcysteine synthetase (γ-ECS). GSH synthetase (GSH-S) then connects the resulting γ-glutamylcysteine with glycine to form GSH. GSH synthesis is regulated by the supply of the constituent amino acids and by feedback inhibition of γ-ECS by GSH (Hell and Bergmann 1990; Rennenberg 1997; May et al. 1998; Noctor et al. 1998b). Overexpressing bacterial gene for γ-ECS in poplar hybrid *Populus tremula* × *P. alba* either in the cytosol (Foyer et al. 1995; Noctor et al. 1996; Arisi et al. 1997) or in the chloroplast (Noctor et al. 1998a) increased foliar and root GSH concentration (Strohm et al. 1995; Noctor et al. 1996, 1998a; Arisi et al. 1997; Herschbach et al. 2000). Overexpression of GSH-S did not affect foliar GSH concentrations, thus confirming the major role of γ-ECS in the control of GSH synthesis. Since in the γ-ECS overexpressing poplars Cys and Met concentrations were not diminished either in the leaves or in the roots (Herschbach et al. 2000) sulfate reduction and Cys formation must be adjusted to the higher demand for GSH synthesis.

The third amino acid constituting GSH, glycine, is produced through photorespiration and is limiting for GSH synthesis in the dark (Noctor et al. 1997a, b, 1999). Furthermore, overexpression of γ-ECS in the chloroplast, but not in the cytosol causes higher foliar concentrations of valine, leucine, isoleucine, tyrosine, and lysine (Noctor et al. 1998a). Since these amino acids are built predominantly in the chloroplasts, it seems that overexpression of γ-ECS in the chloroplast also changed the nitrogen metabolism. Consequently, transgenic poplars manipulated in GSH synthesis represent essential tools to study not only the regulation of sulfate assimilation but also interactions between nitrogen and sulfur metabolism.

Concentration of GSH increased in the phloem of poplars overexpressing γ-ECS, confirming its role as the major transport form of reduced sulfur (Herschbach et al. 1998). The observed linear correlation of the GSH concentration in the phloem with that in the leaves and the roots suggests that either GSH synthesis in the leaves is restricted by its export or phloem transport is determined by GSH synthesis in the leaves (Herschbach et al. 2000).

Both reduced sulfur, mainly as GSH, and sulfate are transported in the phloem to the roots (Herschbach and Rennenberg 2001b). Correlation analysis revealed that the sulfate-to-GSH ratio may be able to control sulfate uptake and loading into the xylem under both enhanced (γ-ECS overexpressing poplar) and decreased (H_2S fumigation) sulfate demand in poplar. In addition to the greater sulfate uptake the poplars overexpressing γ-ECS showed an enhanced GSH concentration in xylem sap (Herschbach et al. 2000). Because GSH does not exchange between the phloem and the xylem in deciduous trees, GSH in the xylem must have originated from GSH synthesis in roots (Herschbach and Rennenberg 1997, 2001b). Elucidation of the contribution of sulfate reduction in the roots to the reduced sulfur budget of the whole plant and its regulation by reduced sulfur from the shoot demands new transgenic poplar lines manipulated in sulfur metabolism, e.g. in the key enzyme of sulfate reduction, adenosine 5'phosphosulfate reductase.

Transgenic trees in stress physiology

Oxidative stress

AOS, i.e. singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot -}$), are formed in plant cells due to exposure to envi-

Table 2 List of transgenic trees manipulated in genes involved in stress defense

Tree species	Gene manipulated	Origin of the coding sequence	Compartment	Promoter	Orientation	Literature
Liriodendron tulpifera	Mercuric reductase (merA)	*Escherichia coli*	Cytosol	CaMV 35S	Sense	Rugh et al. (1998)
Populus tremula × *P. alba*	Fe superoxide dismutase (FeSOD)	*Arabidopsis thaliana*	Chloroplast	CaMV 35S	Sense	Arisi et al. (1998)
Populus tremula × *P. alba*	Chalcone synthase (chs)	*Petunia*	Cytosol	CaMV 35S	Sense	Nicolescu et al. (1996)
Juglans nigra × *J. regia*	Chalcone synthase (chs)	Walnut		CaMV 35S	Antisense	Euch et al. (1998)

ronmental stress caused by light, chilling, drought, nutrient deficiency, wounding, pathogens, ozone, herbicides, or sulfur dioxide (Polle and Rennenberg 1993). AOS can be detoxified directly by radical scavengers such as ascorbate, GSH, carotenoids, or tocopherol, or enzymatically by superoxide dismutase (SOD), catalase, or various peroxidases (Noctor et al. 1998b; May et al. 1998). The most abundant scavenger as well as peroxidase substrate is ascorbate. Ascorbate can be regenerated from its oxidized form, dehydroascorbate, in the ascorbate–GSH cycle, which is active both in the cytosol and chloroplast (Polle and Rennenberg 1993; Noctor and Foyer 1998). The concentration of oxidized glutathione (GSSG) or its ratio to reduced GSH is a measure of the extent of oxidative stress (Foyer and Rennenberg 2000). It was, therefore, expected that increasing the GSH concentration or reduction status would increase plant capacity to cope with oxidative stress. Again, transgenic poplars manipulated in GSH synthesis or overexpressing enzymes involved in detoxification of AOS proved to be excellent tools in addressing physiology of stress resistance in plants (Table 2).

Surprisingly, overexpression of γ-ECS in transgenic poplar, resulting in increased GSH content, did not increase resistance against the herbicide paraquat, although this was enhanced in wild-type poplar upon simultaneous feeding with GSH (Will 1998). Overexpression of glutathione reductase (GR) in the chloroplast, which increased foliar GSH content and reduction state, also did not improve tolerance to paraquat but the plants were less susceptible to photoinhibition (Foyer et al. 1995). On the other hand, increased ascorbate peroxidase and GR activity in transgenic cotton overexpressing MnSOD in chloroplasts (Payton et al. 1997) and overexpression of GR in the chloroplasts of tobacco resulted in a slightly increased resistance against both high light and paraquat triggered damage (Foyer and Rennenberg 2000). It seems, therefore, that the capacity to regenerate GSH by GR activity may be more important for protection against oxidative stress than enhanced foliar GSH concentration (Foyer et al. 1995).

Clearly, manipulation of other enzymes involved in the detoxification of AOS, such as SOD, may potentially improve stress resistance. Beside transgenic tobacco and cotton (Rennenberg and Polle 1994; Payton et al. 1997) transgenic poplars overexpressing FeSOD in the chloroplast (Table 2) were produced but the increased SOD activity did not affect photoinhibition of photosystem II. Apparently, SOD is not limiting for protection against this stress (Tyystjärvi et al. 1999). Under certain conditions, e.g. at low intercellular CO_2 concentrations, overexpression of FeSOD prevented the photochemical chlorophyll *a* fluorescence quenching and stimulated the superoxide producing Mehler reaction (Arisi et al. 1998). It is thus plausible to conclude that the function of GSH in detoxification of AOS resulting from Mehler reaction and photoinhibition may be different.

Whereas in the chloroplast AOS derive from photoinhibition and through the Mehler reaction, ozone exposure results in AOS production at the cell surface, i.e. at the plasma membrane or in the apoplast (Laisk et al. 1989; Polle 1998). Investigations with transgenic poplars overexpressing γ-ECS or GSH-S in the cytosol or GR in the cytosol or in the chloroplast clearly demonstrated that increasing these foliar enzyme activities was not sufficient to improve tolerance to chronic or acute ozone exposure (Will et al. 1997; Strohm et al. 1999). Moreover, these studies revealed that ozone sensitivity was dependent on leaf age, with highest ozone tolerance in young leaves, and thus probably controlled by leaf development. Due to the absence of a correlation between ozone resistance and the foliar GSH content, cell-type-specific GSH concentrations and/or differences in the subcellular distribution might be more important parameters for the increase in ozone (and other oxidative stress) tolerance (Foyer and Rennenberg 2000).

Stress by heavy metals and pesticides

Some heavy metals are essential micro-nutrients for plants (Cu, Ni, Zn) whereas others have no or an unknown physiological role (Pb, Hg, Cd). Plants have developed different mechanisms to keep the intracellular metal concentrations below the toxic level such as immobilization, exclusion, and chelating (Rauser 1999). Most heavy metals can be chelated by Cys-rich proteins, metallothioneins, or by phytochelatins (PCs), small polypeptides consisting of repeating γ-EC units (Rauser 1999; Cobbett 2000).

PCs are synthesized from GSH by PC synthase (Grill et al. 1989). The sulfhydryl groups of Cys residues bind

the heavy metal ions and the resulting complexes are excreted into the vacuole. Heavy metals induce the synthesis of PCs via post-translational activation of PC synthase and induction of γ-ECS (Rauser 1999; Cobbett 2000; Foyer and Rennenberg 2000). In *Brassica juncea* overexpression of the bacterial gene for γ-ECS resulted in enhanced PC concentration and increased Cd tolerance (Zhu et al. 1999a, b). Since exposure of the γ-ECS overexpressing poplars to Cd also increased PC content, GSH synthesis seems to limit PC production and, consequently, heavy metal detoxification (Rennenberg and Will 2000). The exposure to Cd also induced activities of malic enzyme and isocitrate dehydrogenase, corresponding with the increased demand for NADPH for reduction of GSSG by GR (Arisi et al. 2000). Surprisingly, despite the increased PC synthesis, the γ-ECS overexpressing poplars were not more tolerant to high concentrations of Cd (Rennenberg and Will 2000). The observed differences between *B. juncea* and poplar might be caused by general differences between herbaceous and deciduous plants or related to the specific S-metabolism of *B. juncea* (glucosinolate synthesis). However, since Cd accumulation correlated with PC levels, poplars overexpressing γ-ECS accumulated more Cd. Apparently, increased Cd binding due to higher PC synthesis may diminish the active intracellular Cd pool in the roots, thus resulting in higher uptake of Cd (Rennenberg and Will 2000).

Differences in herbicide tolerance are often based on the plant capacity to detoxify the herbicide, e.g. through the GST reaction and subsequent excretion of the conjugate into the vacuole (Edwards et al. 2000). It is thus not surprising that growth of transgenic poplars overexpressing γ-ECS in the cytosol or in the chloroplast was less reduced upon treatment with chloroacetanilide herbicides than that of the wild-type (Gullner et al. 2001). The reduced herbicide effects correlated with increased GST activity revealing once more the important role of GSH in plant defence.

Phytoremediation, the use of plants to remove contaminants from soil or water, is a promising approach to cope with environmental pollution (Salt et al. 1998). Trees might be more suitable for phytoremediation than herbaceous plants because of their high biomass and long generation cycles (discussed in Kopriva and Rennenberg 2000). Accordingly, overexpression of the bacterial mercuric reductase in yellow poplar (*Liriodendron tulipifera*) resulted in transgenic plants that were resistant to toxic levels of mercuric ions and able to release elemental mercury (Rugh et al. 1998). Also, the transgenic poplars overexpressing γ-ECS are attractive for phytoremediation of heavy metals and herbicides due to the higher uptake capacity for Cd and increased GST activity, respectively (Rennenberg and Will 2000; Gullner et al. 2001).

Secondary compounds

Many secondary plant compounds, e.g. glucosinolates, alkaloids, or flavonoids, are involved in plant stress response. Flavonoids play an important role in defence against pathogens, protection from UV radiation, and in rhizogenesis (Curir et al. 1990). In order to characterize their role in rhizogenesis, the activity of chalcone synthase, a key enzyme in flavonoid biosynthesis, was decreased in walnut by an antisense strategy (Euch et al. 1998). The transgenic plants were characterized by very low levels of flavonoids correlating with enhanced adventitious root formation. Auxin accumulation was not varied in the transgenic lines. The plants were, however, more sensitive to exogenous auxin applications, leading to leaf and root necrosis. It seems that since flavonoids regulate auxin transport, the transport of sucrose required for root formation is also affected (Haissig 1990). On the other hand, overexpression of chalcone synthase led to increased flavonoid levels in cortical and peripheral tissues of stems; nevertheless increased protection against pathogens in these plants remains to be demonstrated (Nicolescu et al. 1996).

Transgenic trees in nitrogen metabolism

To our knowledge only one transgenic tree manipulated in nitrogen metabolism has been reported. Overexpression of a *Pinus pinaster* glutamine synthetase under control of the CaMV35 promoter in poplar (*Populus tremula* × *P. alba*) resulted in increased total soluble protein and chlorophyll contents and led to significantly better growth (Gallardo et al. 1999). These experiments thus confirm that glutamine synthetase has a high control over ammonium assimilation (Lam et al. 1996). Since nitrogen availability in the soil might be a limiting factor for plant growth, an increased efficiency of nitrogen utilization may improve tree growth, development, and stress resistance.

Plant hormones

Plant hormones, such as auxin (indole-3-acetic acid, IAA), gibberellins (GA), cytokinins, or abscisic acid (ABA), are involved in the regulation of plant growth and development (Kende and Zeevaart 1997). Since they also influence wood formation (Little and Savidge 1987) the manipulation of endogenous hormone levels in stem tissues of trees is of great interest. Analysis of genetically modified trees helped to establish the specific roles of plant hormones in tree physiology (Table 3).

IAA is crucial for the structure and activity of vascular cambium, in particular cambial cell division. The increase of IAA concentration via simultaneous overexpression of *iaaM* (Trp-2-mono-oxygenase) and *iaaH* (indole-3-acetamide hydrolase) genes for IAA synthesis from *Agrobacterium tumefaciens* T-DNA in *Populus tremula* × *P. tremuloides* resulted in alterations in growth, development, and wood formation (Tuominen et al. 1995). The lower rate of cambial cell division and the longer duration of expansion resulted in decreased xylem

Table 3 List of transgenic trees manipulated in hormone contents

Tree species	Gene overexpressed	Origin of the coding sequence	Compartment	Promoter	Orientation	Literature
Populus tremula × *P tremuloides*	Trp-2-mono-oxygenase (*iaaM*), indole-3-acetamine hydrolase (*iaaH*), *iaaM* + *iaaH*	*Agrobacterium tumefaciens*	Cytosol	Mannopine synthase (*iaaM*) CaMV 35S (*iaaH*)	Sense	Tuominen et al. (1995)
Populus tremula × *P tremuloides* expressing *iaaH*	Trp-2-mono-oxygenase (*iaaM*)	*Agrobacterium tumefaciens*	Cytosol	RolC	Sense	Tuominen et al. (2000)
Populus tremula × *P tremuloides*	GA-20-oxidase	*Arabidopsis thaliana*	Cytosol	CaMV 35S	Sense	Eriksson et al. (2000)
Populus tremula × *P tremuloides*	Phytochrome A (phyA)	Oat	Cytosol	CaMV 35S	Sense	Olsen et al. (1997)
Malus domestica	Phytochrome B (phyB)	*Arabidopsis*	Cytosol	CaMV 35S	Sense	Holefors et al. (2000)
Populus nigra	Homeobox *OSH1*	Rice	Cytosol	CaMV 35S	Sense	Mohri et al. (1999)
Populus tremula × *P. alba*	Isopentenyl transferase (ipt)	*Agrobacterium tumefaciens*	Cytosol	Ipt	Sense	Von Schwartzenberg et al. (1994)
Populus sieboldii × *P. grandidentata*	Isopentenyl transferase (ipt)	*Agrobacterium tumefaciens*	Cytosol	CaMV 35S	Sense	Ebinuma et al. (1997)
Populus tremula × *P tremuloides*	*rolC*	*Agrobacterium rhizogenes*	Cytosol	CaMV 35S	Sense	Fladung et al. (1996); Nilsson et al. (1996a)
Populus tremula × *P tremuloides*	*rolC*	*Agrobacterium rhizogenes*	Cytosol	rbcS	Sense	Fladung et al. (1997)
Populus tremula	*rolB* + *rolC*	*Agrobacterium rhizogenes*	Cytosol	rolC	Sense	Tzfira et al. (1998b, 1999)
Malus domestica	*rolB*	*Agrobacterium rhizogenes*	Cytosol	rolB	Sense	Welander et al. (1998); Zhu et al. (2001)
Malus domestica	*rolA*	*Agrobacterium rhizogenes*	Cytosol	rolA	Sense	Holefors et al. (1998)
Poncirus trifoliata	*rolC*	*Agrobacterium rhizogenes*	Cytosol	CaMV 35S	Sense	Kaneyoshi and Kobayashi (1999)
Actinidia deliciosa	*rolA, rolB* +*rolC*	*Agrobacterium rhizogenes*	Cytosol	rolA, rolB + rolC	Sense	Rugini et al. (1991)
Pyrus communis	*rolC*	*Agrobacterium rhizogenes*	Cytosol	rolC	Sense	Bell et al. (1999)

production and was related to a lower peak level and a wider radial distribution of IAA within the cambial meristem (Tuominen et al. 1997). Since the overall growth of the transgenic lines was also altered, in the next study the *iaaM* gene was expressed in poplar under control of a cambium-specific *rolC* promoter (Tuominen et al. 2000). The *rolC* promoter from *Agrobacterium rhizogenes* exhibits tissue-specific changes during the annual growth cycle and dormancy and is also sensitive to chilling and sucrose (Nilsson et al. 1996b). However, although the IAA concentration in the cambial region was 35–40% higher than in wild-type plants, no changes in the radial distribution pattern of IAA and thus no changes in developmental pattern or cambial growth rate were observed (Tuominen et al. 2000). It seems therefore that it is not the absolute amount of IAA but its distribution pattern that regulates the development of secondary xylem.

GAs are a group of plant diterpene compounds that influence shoot elongation, leaf expansion and shape, flowering, seed germination, and, in trees, differentiation of xylem fibres (Kende and Zeevaart 1997). Therefore, GA 20-oxidase, the key enzyme controlling GA biosynthesis, was overexpressed in *Populus tremula* × *P. tremuloides* resulting in faster growth and increased biomass in the transgenic plants (Eriksson et al. 2000). The stem diameter increased as a result of increased number and length of xylem fibres. On the other side, root initiation was negatively affected in young plantlets of the transgenic lines; the effect disappearing at later growth stages. These results show that manipulation of GA levels may lead to trees that grow faster and produce more biomass but also provide tools to study the molecular mechanisms behind GA control of growth and development.

Isopentenyl transferase (IPT) is an important enzyme in the biosynthesis of cytokinins. Cytokinins stimulate organogenesis and are necessary for regeneration of plants from cell cultures or calli. The overexpression of

Table 4 List of transgenic trees manipulated in flowering

Tree species	Gene manipulated	Origin of the coding sequence	Compartment	Promoter	Orientation	Literature
Populus tremula × *P. tremuloides*	*LEAFY*	*Arabidopsis thaliana*	Cytosol	CaMV 35S	Sense	Weigel and Nilsson (1995)
Populus tremula × *P. alba*, *P. tremula* × *P. tremuloides*, *P. trichocarpa* × *P. deltoides*	*LEAFY*	*Arabidopsis thaliana*	Cytosol	CaMV 35S	Sense	Rottmann et al. (2000)
Populus tremula × *P. alba*, *P. tremula* × *P. tremuloides*, *P. trichocarpa* × *P. deltoides*	*PTLF* (*LEAFY* homolog)	*Populus trichocarpa*	Cytosol	CaMV 35S	Sense, antisense	Rottmann et al. (2000)
Citrus sinensis × *Poncirus trifoliata*	*LEAFY*	*Arabidopsis thaliana*	Cytosol	CaMV 35S	Sense	Peña et al. (2001)
Citrus sinensis × *Poncirus trifoliata*	*APETALA1*	*Arabidopsis thaliana*	Cytosol	CaMV 35S	Sense	Peña et al. (2001)

A. tumefaciens ipt gene in poplar resulted in transgenic plants with strongly altered phenotypes: low apical dominance, branching shoots, short internodes, and inability to form roots. plants contained 5–17 times higher levels of zeatin and other cytokinins (Von Schwartzenberg et al. 1994). Most importantly, shoots differentiated from transgenic calli even in the absence of phytohormones in the medium. The *ipt* gene can thus be used as a selectable marker for transformation and in combination with transposable element *Ac*, which enables loss of the chimeric *ipt* gene, to generation of marker-free transgenic plants (Ebinuma et al. 1997).

Cytokinin levels in plants were also modified by overexpression of *rol* genes of *Agrobacterium* leading to morphological changes in the transgenic plants (Spena et al. 1987). Transgenic poplars (*Populus tremula* × *P. tremuloides*) overexpressing the *rolC* gene from *A. rhizogenes* were characterized by reduced IAA, changes in cytokinin composition, and in GA to ABA ratios dependent on the tree region, resulting in dwarfed phenotype, loss of apical dominance, and premature bud and leaf development (Nilsson et al. 1996a; Fladung et al. 1997). The wood structure in these transgenic trees was not significantly different but initiation of wood production was delayed from that of wild-type poplars. Moreover, latewood lacked the thick-walled fibres and discoloration, and the formation of tyloses was slightly altered (Grünwald et al. 2000). When both *rolB* and *rolC* were expressed under control of their native promoters the apical dominance was still lost; the transgenic plants, however, displayed an enhanced growth rate and delayed winter dormancy (Tzfira et al. 1999). In contrast, transgenic fruit trees overexpressing *rol* genes showed a reduced internode length and biomass production (Holefors et al. 1998; Bell et al. 1999; Kaneyoshi and Kobayashi 1999; Zhu et al. 2001). Nevertheless, all trees overexpressing *rol* genes also exhibited extensive root formation in a hormone-free medium, larger root surface area, as well as rapid adventitious root formation which together might result in shorter propagation times (Tzfira et al. 1998b). These characteristics are of major importance for fruit tree breeders, since dwarfing rootstocks, which are used to increase productivity, are difficult to root from cuttings. Indeed, overexpression of *rol*B gene from *A. rhizogenes* in kiwi trees or apple rootstocks significantly increased rooting on a hormone-free medium (Rugini et al. 1991; Kaneyoshi and Kobayashi 1999; Zhu et al. 2001). Although the overexpression of *rolB* in apple resulted in increased sensitivity to auxin, elevated auxin was necessary to induce rooting in vitro (Welander et al. 1998).

In addition, the rice homeobox gene *OHS1* also affects the hormone metabolism and thus induces morphological aberrations. Overexpression of *OSH1* gene in poplars resulted in morphological changes such as slender leaves, dwarf plants, or loss of apical dominance (Mohri et al. 1999). Evidently, the manipulation of hormone metabolism is an effective tool to study the regulatory mechanisms of tree development and wood formation.

Transgenic trees in reproduction biology

Trees usually have a long juvenile growth period before they reach their reproductive phase. Flower development in poplar also differs significantly from that of herbaceous plants (Boes and Strauss 1994). The male and female flowers develop on separate trees from axillary inflorescences. Instead of four concentric whorls of organs poplar flowers have only two. Nevertheless, homologues of genes involved in flower development in *Arabidopsis* and *Antirrhinum* were identified from several tree species: *LEAFY* and *Floricaula* from *Pinus radiata*, *Eucalyptus*, and poplar (Mouradov et al. 1998; Southerton et al. 1998; Rottmann et al. 2000), *AGAMOUS* from black

spruce (Rutledge et al. 1998), and *AP1* from *Eucalyptus* (Kyozuka et al. 1997). Genetic manipulations of flowering genes are mainly aimed to shorten flowering and generation time (Table 4). In citrus expression of *Arabidopsis* genes *LEAFY* and *APETALA1* induced flowering within the first year. The shortening of the juvenile period was stable and also observed in the zygotic and nucellar-derived seedlings demonstrating the stability of the trait (Peña et al. 2001). In poplar, however, the influence of flowering genes is more complex. The expression pattern of the endogenous *LEAFY* homologue, *PTLF*, in poplar differed significantly from that of *LEAFY* in *Arabidopsis*, since *PTLF* was also expressed in vegetative tissues (Rottmann et al. 2000). Overexpression of *LEAFY* from *Arabidopsis* in poplar affected shoot meristems and accelerated flower development (Weigel and Nilsson 1995). On the other hand, overexpression of *PTLF* from *Populus trichocarpa* in several *Populus* species induced precocious flowering in only two of 19 transgenic lines, although in *Arabidopsis* overexpressing *PTLF* flowering was accelerated (Rottmann et al. 2000). The transgenic *Populus* lines produced anthers rather than carpels on originally female flowers, indicating the original role of *LEAFY* in male flowering (Frohlich and Parker 2000). Transgenic poplar lines with high levels of *PTLF* overexpression, however, exhibited abnormal vegetative morphology, such as increased ramification of branches from the current year's growth in their third year. In theory, antisense expression of the *PTLF* should abolish or at least delay the formation of flowers in transgenic poplars. The evaluation of such plants is, however, difficult due to the fact that poplars need 5 or more years before they are reproductively mature and changes in flowering can be detected. The increased knowledge about the control of flower development in trees thus opens up a way to produce sterile plants, with high impact for the biological safety of transgenic plants release (Strauss et al. 1995).

The cause of transition from vegetative to reproduction growth is still not clear, although genes such as *LEAFY* and environmental factors seem to be involved. *LEAFY* and other flower controlling genes may interact with genes controlling responses to day length (Haughn et al. 1995). Moreover, the growth cycle of trees is dependent on perception of environmental signals, primarily light. The exact role of different light photoreceptors is, however, not known yet. The function of phytochrome A, which is known to control flowering in *Arabidopsis*, in the detection of photoperiod in trees was studied by overexpression of oat phytochrome A gene in poplar (Olsen et al. 1997). The transgenic trees did not reduce the levels of GA and IAA under short day conditions and thus did not stop growing even at 6 h photoperiod. It seems, therefore, that the level of phytochrome A expression affects plant hormone metabolism and might thus be responsible for different photoperiodic responses of photoperiodic ecotypes. In addition *Malus domestica* trees overexpressing phytochrome B could be a useful tool to analyse light- and hormone-regulated processes (Holefors et al. 2000).

Conclusions

The methods of molecular biology have a great impact on plant science, including investigations of trees. Trees possess several specific physiological characteristics such as long life cycles from a few to many decades and centuries, wood production, phases of dormancy combined with storage processes, or a long distance between the shoot and the roots. Biotechnology thus plays a major role in attempts to explore and exploit tree genetic variation and to improve tree characteristics. Genetically modified trees are, however, not only products for agroforestry to increase wood production and quality. As demonstrated in this review, transgenic trees are outstanding tools for studying aspects specific of tree physiology, but also general plant physiological processes. Transgenic trees expressing genes involved in hormone biosynthesis provide knowledge on the role of genetic and hormonal factors in tree growth and development, but also provide material to study the general role of these substances in plants. Our knowledge on regulation of sulfur nutrition and GSH synthesis in higher plants was significantly improved by investigations with transgenic poplars. Although trees are not as easy for laboratory use as herbaceous plants due to their slower growth, large size, and long reproduction cycles, they are increasingly exploited for investigations of other than tree-specific problems. Transgenic poplar, as the most common model system for perennial plants, will become more useful to demonstrate differences and similarities to annual plants' physiology.

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