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Lignin characteristics and ecological interactions of PtCOMT-modified silver birch

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Academic dissertation

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ABSTRACT

The 35S- and UbB1-*PtCOMT* genes were transferred by biolistic bombardment into silver birch, *Betula pendula* Roth, and the effects on syringyl (S) lignin unit synthesis were investigated. The transgenes were stably integrated into the *B. pendula* genomes, and their variable expression was observed. In the 35S-PtCOMT lines, a reduced syringyl/guaiacyl (S/G) ratio and incorporation of abnormal 5-OH-G units into lignin were found. This was apparently due to the RNA interference (RNAi)-based suppression of the *COMT* gene, leading to reduced S lignin content in stems, leaves and roots. This work supports the essential role of COMT for S unit synthesis and the current view on lignin biosynthesis in woody angiosperms. The unchanged morphology and growth characteristics of the 35S-PtCOMT modified *B. pendula* lines also indicate that plants are able to tolerate a large variation in the lignin S/G ratio.

PtCOMT-promoter-GUS (β-glucuronidase) modified *B. pendula* lines were produced in order to investigate the expression pattern of the *PtCOMT* gene. The main activity during the growing season was present in the new xylem and lignified phloem fibres. Our results also suggest that COMT plays a role in tension wood formation but not in the response to wounding.

In *in vitro* studies focusing on the potential ecological interactions between PtCOMT-modified *B. pendula* lines and insect herbivores and ectomycorrhizal fungi (ECM), the role played by birch clone or transgenic line was found to be important. The preferential leaf quality to insect herbivores was not directly related to the PtCOMT-modified lignin, but resulted potentially from indirect factors such as a changed growth rhythm. All PtCOMT lines were able to form ECM with *P. involutus*, but the specific ECM characteristics, e.g. formation of a Hartig net (HN), differed from those of the control. The extent to which the different ECM characteristics of specific PtCOMT lines are related to lignin modification or unintended effects of transgenes remains to be solved.

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Imatra, November 2007 Heidi Tiimonen

LIST OF ORIGINAL ARTICLES

This thesis is based on the following articles and manuscript, which are referred to in the text by their Roman numerals:

I. Aronen, T., Tiimonen, H., Tsai, C.-J., Jokipii, S., Chen, X., Chiang, V. and Häggman, H. 2003. Altered lignin in transgenic silver birch (*Betula pendula)* expressing *PtCOMT* gene. In: Espinel, S., Barredo, Y. & Ritter, E. (eds.) Sustainable forestry, wood products & biotechnology. DFA-AFA Press, Vitoria-Gasteiz, Spain. p. 149-161.

II. Tiimonen, H., Häggman, H., Tsai, C.-J., Chiang, V. and Aronen, T. 2007. The seasonal activity and the effect of mechanical bending and wounding on the PtCOMT promoter in *Betula pendula* Roth. Plant Cell Reports 26: 1205-1214.

III. Tiimonen, H., Aronen, T., Laakso, T., Saranpää, P., Chiang, V., Ylioja, T., Roininen, H. and Häggman, H. 2005. Does lignin modification affect feeding preference or growth performance of insect herbivores in transgenic silver birch (*Betula pendula* Roth)? Planta 222: 699-708.

IV. Tiimonen, H., Aronen, T., Laakso, T., Saranpää, P., Chiang, V., Ylioja T., Häggman, H. and Niemi K. *Paxillus involutus* forms ectomycorrhizal symbiosis with PtCOMT-modified Betula pendula *in vitro*. (Submitted for publication)

V. Häggman, H., Niemi, K., Tiimonen, H., Ylioja, T. and Chiang, V. 2006. Environmental aspects of lignin modified trees. In: Fladung, M. & Ewald, D. (eds.) Tree transgenesis: Recent developments. Springer-Verlag, Berlin, Heidelberg, Germany. p. 105-122.

AUTHOR´S CONTRIBUTION

Paper I: The author was responsible for the collection and molecular analysis of the material as well as for the measurement of growth characteristics.

Paper II: The author was responsible for collection of the research data, molecular analyses and writing the manuscript.

Papers III: The author was the corresponding author, and thus responsible for the research planning, collection of research data, molecular analyses and writing the manuscript.

Paper IV: The author was the corresponding author, and thus responsible for the research planning, collection of research data, molecular analyses and writing the manuscript.

Paper V: The author was responsible for writing the paragraph “Environmental aspects of processing lignin-modified trees in the pulp and paper industry”.

CONTENTS

INTRODUCTION

From genomics to biotechnological approaches to forest trees

Genetic and molecular tools have considerably advanced our understanding of the genes associated with complex biological systems in both herbaceous (*Arabidopsis*) and woody (*Populus*) model species (Boerjan 2005, Merkle & Nairn 2005, Nehra et al. 2005). These complex systems include wood formation, wood quality, meristem identity and the reproductive control of trees (Bhalerao et al. 2003). Currently, the only forest tree species for which the genome has been fully sequenced is *Populus* (Brunner et al. 2004, Tuskan et al. 2004, IPGC 2007). However, a lot of sequence information has been gathered from EST (expressed sequence tag) gene identification projects on e.g. *Populus*, *Betula* spp., *Eucalyptus* and several *Pinus* and *Picea* species (Merkle & Nairn 2005, Nehra et al. 2005). The achievements of the genomic and functional genomic era have also been advantageous for forest tree biotechnology. Early flowering via transgenic approach has been expected to speed up tree breeding (Boerjan 2005, Merkle & Nairn 2005, Nehra et al. 2005, Plomion et al. 2005, Böhlenius et al. 2006). On the global scale, the production of trees with improved characteristics is expected to help meet the growing need for wood and wood-related products (FAO 2005), and hence to diminish the pressure on the domestication of natural forest resources in the future (Boerjan 2005, Merkle & Nairn 2005, Nehra et al. 2005).

Several conifer and angiosperm tree species have been genetically transformed, with the major focus on traits including wood quality, insect and disease resistance and abiotic stress tolerance (Boerjan 2005, Merkle & Nairn 2005, Nehra et al. 2005). Transgenic trees with various genetically modified (GM) traits have also been tested in field conditions, e.g. insect-resistant *Populus nigra* (Hu et al. 2001, Lin et al. 2006), and *Picea glauca* (Lachance et al. 2007), several herbicide-resistant *Populus hybrids* (Meilan et al. 2002), lignin-modified *Populus tremula* x *Populus alba* (Pilate et al. 2002), fungal disease resistant *Betula pendula* (Pasonen et al. 2004), sterile *B. pendula* (EC 2007) and sterile *P. tremula* x *P. alba* (Wei et al. 2006). Field testing is generally considered to be important for assessment of the potential environmental effects of genetic modification in organisms such as trees that are characterised by e.g. longevity, wind pollination, and multiple ecological interactions in forest ecosystems. Up to now, the commercialization of GM trees has lagged well behind that of GM crop plants. The area of cultivated, commercialized GM crop plants in 2006 covered 102 million. hectares (James 2006), whereas there are only two commercially cultivated GM trees, i.e. the virus-resistant papaya fruit tree in Hawaii (Ferreira et al. 2002) and insect-resistant poplar cultivations in China (FAO 2004).

The ability to modify lignin as a part of cell wall modification is an important area of research in attempts to improve the utilization of plant biomass as a renewable source for sustainable development (Boerjan 2005, Chiang 2006, Higuchi 2006, Li et al. 2006). The development of trees with improved wood quality through modification of the genes involved in lignin biosynthesis could be important for the improved end use of wood material (Chiang 2006, Higuchi 2006). In chemical wood pulping, lignin is the main factor hindering the effective utilisation of cellulose fibres, from which it needs to be separated by costly and pollutant-generating processes (Chiang 2002, Baucher et al. 2003, Boerjan 2005). Genetic modifications resulting in increased delignification (through a modified lignin content and/or chemical composition) could thus be highly beneficial at both the economical and environmental scale (Baucher et al. 2003, Boerjan 2005, Chiang 2006). On the other hand, taking into account the positive correlation between the lignin content and heating value of

wood (White 1987, Baucher et al. 1998) and the enormous amount of wood which is annually consumed in fuel production (c. 1200 million m^3) (FAO 2005), an improved fuel value due to its enriched lignin content might be an alternative option worth considering.

Lignin

Occurrence, functions, composition and natural variation

Lignin is a phenolic heteropolymer associated with cellulose and hemicellulose in the secondary cell walls of vascular elements, fibres and sclereids in vascular plants. Lignin, especially that in trees, accounts for an enormous reservoir of organic carbon in the biosphere. Lignin deposition occurs after the completion of cell growth and when the three layers of secondary cell walls, the outer (S1), middle (S2) and inner (S3), are assembled during thickening of the secondary cell wall. Lignin deposition begins at the cell corners in the primary cell wall and in the middle lamella when S1 formation has initiated. After the deposition of polysaccharides, lignification then proceeds in two distinct stages through the S2 and S3 layers (Baucher et al. 1998, Boerjan et al. 2003).

The ability to synthesize lignin has created the necessary conditions for the terrestrial lifestyle of plants. The main function of lignin is to provide structural integrity of the cell walls, which is crucial for woody plants with a high need for structural support and stem rigidity. As a hydrophobic molecule, lignin waterproofs the cell walls and is hence elementary for the transport of water and solutes in the xylem (the vascular system) (Wardrop 1971, Baucher et al. 1998, Boerjan et al. 2003). The insolubility and complex structure of lignin polymers lead to high resistance against microbial degradation, and they thus play an important role in plant defence (Vance et al. 1980, Walter 1992, He et al. 2002, He & Wolyn 2005). From the human point of view, however, lignin has a negative impact on wood pulping because the need to extract lignin from cellulose fibres both decreases the yield of cellulose and is an economically and environmentally costly process (Baucher et al. 2003).

In gymnosperms lignin is composed mainly of guaiacyl (G) units, whereas in angiosperms syringyl (S) units are also involved. The third phenylpropanoid unit, p-hydroxyphenyl (H), is mostly involved in grass (Gramineae) lignins (Boerjan et al. 2003, Higuchi 2006), but it is also a minor element both in gymnosperm and angiosperm lignin (e.g. Cabané et al. 2004). The G and S units differ in the degree of methylation of the 5-position of the aromatic ring. Owing to the methylated 5-position, S units are able to form β-O-4 inter-unit linkages, which are the most common and easily cleavable linkage type between the lignin monomers. In the G units, on the other hand, the 5-position is available for the resistant inter-unit linkages, such as β-5, 5-5 and 5-O-4 (reviewed by Boerjan et al. 2003). The monolignol composition is directly proportional to the relative abundance of different linkages and, therefore, to the chemical solubility of lignin. Consequently, the ratio of syringyl to guaiacyl units in lignin is the major factor responsible for the faster delignification rate of hardwoods compared to softwoods during chemical delignification, such as the widely used kraft pulping (Chang & Sarkanen 1973, Chiang & Funaoka 1990).

Lignin quantity and quality vary naturally (Sarkanen & Hergert 1971, Grand et al. 1982, Campbell & Sederoff 1996). Depending on the species of woody plant the lignin content may vary from 15 to 36% of the dry weight of wood (Higuchi 2006). Angiosperm wood is typically composed of vessels, fibres and ray parenchyma cells, and the secondary cell walls of vessels have a higher lignin concentration compared to the fibres and ray parenchyma cells (Baucher et al. 1998, Boerjan et al. 2003). The vessel wall lignins are also enriched with G

units, whereas in the fibre and ray cell wall lignin S units predominate in *Betula* (Fergus & Goring 1970, Hardell et al. 1980, Saka & Goring 1988) and many other hardwood species (Musha & Goring 1975). In general, during secondary cell wall assembly the G units are deposited prior to the S units (Terashima et al. 1986, Bhalerao et al. 2003). The S/G ratio may also change during the development of the plant, and the S unit content of lignin especially has been found to increase with age (Grand et al. 1982). The increased proportion of S monomers is also indicative of tension wood, i.e. the reaction wood specific to angiosperm species that is formed as a result of the mechanical stress of stem bending (Sarkanen & Hergert 1971).

The current view of lignin biosynthesis in deciduous trees

The intensive research on monolignol biosynthesis in recent decades (reviewed e.g. by Higuchi 1985, Baucher et al. 1998, Boerjan et al. 2003, Li et al. 2006) has resulted in an improved understanding of the enzymatic reactions occurring at the hydroxycinnamic ester, aldehyde and alcohol levels, which lead to the synthesis of guaiacyl (G) and syringyl (S) monomers. The current view of monolignol biosynthesis in angiosperms is presented in Figure 1 (modified from Li et al. 2006), and in the following description of the biosynthesis pathway. The gene copy number of the respective enzyme in *Populus* is given in parentheses when available (Tsai et al. 2006). The pathway is initiated by the deamination of phenylalanine to cinnamate by phenylalanine ammonia-lyase (PAL). In the next step, cinnamate 4-hydroxylase (C4H, 2) catalyses the conversion from cinnamate to 4-coumarate. These are either 3-hydroxylated to caffeate by 4-coumarate 3-hydroxylase (C3H, 3) or, alternatively, converted to 4-coumaroyl CoA by 4-coumarate-CoA ligase (4CL, 5) and then to 4-coumaroyl shikimic acid by hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (CST, 6). C3H further catalyses the reaction from 4-coumaroyl shikimic acids to caffeoyl shikimic acids which, in turn, can then be converted into caffeoyl CoA by CST. Caffeates can also be catalysed by 4CL activity to caffeoyl CoAs, which can then be used for the production of feruloyl CoA by caffeoyl-CoA *O*-methyltransferase (CCoAOMT, 6). In the next reaction, feruloyl CoAs are converted to coniferyl aldehyde by cinnamoyl-CoA reductase (CCR).

Coniferyl aldehyde can either be reduced to coniferyl alcohol by coniferyl alcohol dehydrogenase (CAD) giving rise to guaiacyl units, or alternatively be hydroxylated to 5-hydroxyconiferyl aldehyde by coniferyl aldehyde 5-hydroxylase (CAld5H) or, as also designated, ferulate 5-hydroxylase (F5H, 2) in the syringyl monolignol specific pathway. The second metabolic step toward S monolignol biosynthesis is the production of sinapyl aldehydes from 5-hydroxyconiferyl aldehydes by 5-hydroxyconiferyl aldehyde *O*-methyltransferase (AldOMT), or as is also known, by caffeate/5-hydroxyferulate *O*-methyltransferase (COMT, 9) (Bugos et al. 1991). In the final reaction leading to S monolignol biosynthesis, sinapyl aldehydes are reduced to sinapyl alcohol by sinapyl alcohol dehydrogenase (SAD). For lignin polymerisation, G and S monolignols need to be further oxidised through a proposed action of laccases (Gavnholt & Larsen 2002) and peroxidases (Sasaki et al. 2004, Sasaki et al. 2006). As a result of oxidation, phenoxy radicals are produced that are coupled into lignin polymers either randomly (Morreel et al. 2004) or, according to the alternative model, by the action of dirigent proteins (Davin & Lewis 2005).

S lignin formation in angiosperms

The traditionally accepted pathway for the syringyl monolignol biosynthesis in angiosperms is based on the conversion of caffeate to sinapate via the ferulate 5-hydroxylase (F5H)

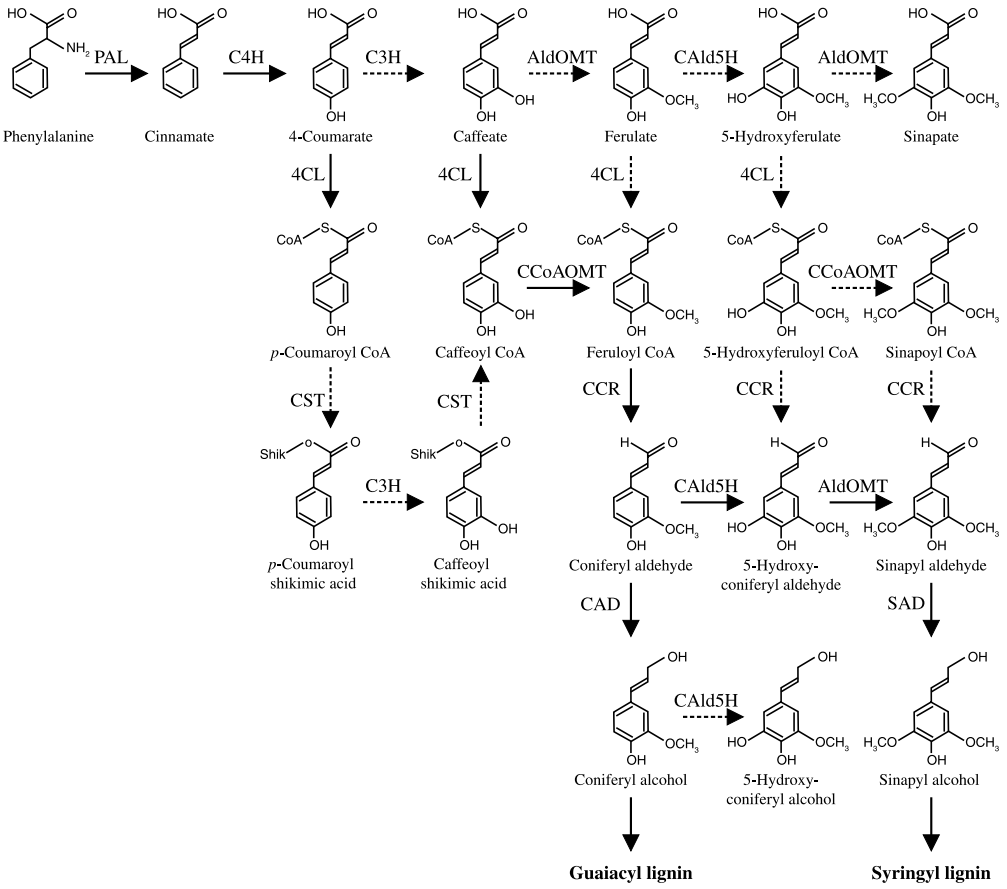

Figure 1. The current view of monolignol biosynthesis in angiosperms (modified from Li et al. 2006). The principal pathways are indicated with solid arrows and possible pathways with dotted arrows. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; 4CL, 4-coumarate-CoA ligase; CST, hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, coniferyl alcohol dehydrogenase; CAld5H, coniferyl aldehyde 5-hydroxylase, or also designated as F5H, ferulate 5-hydroxylase; AldOMT, 5-hydroxyconiferyl aldehyde *O*-methyltransferase, or also designated as COMT, caffeate/5-hydroxyferulate *O*-methyltransferase; SAD, sinapyl alcohol dehydrogenase.

and caffeate *O*-methyltransferase (COMT) enzyme activities (Fig. 1) (Sarkanen & Hergert 1971, Grisebach 1981, Grand 1984, Higuchi 1985, Whetten et al. 1998). Recent findings on the substrate-modulated enzyme activities of F5H (Osakabe et al. 1999) and COMT (Li et al. 2000) have, however, challenged the traditional view by showing that S monolignol biosynthesis in angiosperms may occur independently of both ferulate 5-hydroxylation and caffeate and 5-hydroxyferulate *O*-methylation. Kinetic analysis of the lignifying stem xylem of sweetgum (*Liquidambar styraciflua*) revealed that coniferyl aldehyde is both the substrate for 5-hydroxylation and a noncompetitive inhibitor of ferulate 5-hydroxylation. These results

thus suggest that coniferyl aldehyde 5-hydroxylation would be the dominant reaction leading to syringyl monolignol biosynthesis and, accordingly, the enzyme catalysing this reaction would correspondingly be called coniferyl aldehyde 5-hydroxylase (Cald5H) (Osakabe et al. 1999) instead of F5H (Meyer et al. 1996).

Analyses of the catalytic properties of a recombinant *Populus tremuloides* COMT and of lignifying xylem proteins from ten angiosperm species including *Betula papyrifera* (white birch) and *Betula alleghaniensis* (yellow birch) support the new view on S monolignol biosynthesis in angiosperms (Li et al. 2000). The results demonstrate that 5-hydroxyconiferyl aldehyde would be both the preferred substrate for *O*-methylation and also inhibit the methylation of traditionally accepted substrates, caffeate and 5-hydroxyferulate (Atanassova et al. 1995, Van Doorsselaere et al. 1995). Corresponding to the *O*-methyltransferase activities in these reactions, the enzyme was suggested to be primarily 5-hydroxyconiferyl aldehyde *O*-methyltransferase (AldOMT) (Li et al. 2000) rather than COMT. Taken together, the CAld5H/AldOMT/SAD-mediated reaction sequence seems to divert guaiacyl intermediates toward the synthesis of S monolignol, whereas the previously proposed pathway branch from caffeate to sinapate is not involved in S monolignol biosynthesis in angiosperms.

Transcriptional regulation of lignin biosynthesis genes

The regulation of lignin biosynthesis is a complex issue due to the different regulatory levels that exist, e.g. associations of lignin biosynthesis to primary metabolism via plant growth and carbon allocation (Hu et al. 1999, Rogers et al. 2005, Hancock et al. 2007), the sensitivity of lignin formation to hormonal (Israelsson et al. 2003, Biemelt et al. 2004, Busov et al. 2006, Tokunaga et al. 2006) and sugar (Rogers et al. 2005) signalling and transcriptional regulation of lignin biosynthesis genes (Goicoechea et al. 2005, Andersson-Gunnerås et al. 2006, Marjamaa et al. 2007). Environmental or stress factors, such as ozone (Cabané et al. 2004) or heavy metal (Díaz et al. 2001) exposure, wounding (Lauvergeat et al. 2002, Hawkins & Boudet 2003, Zhao et al. 2005), or pathogen infection (Capellades et al. 1996, Chen et al. 2000) may also induce or alter lignin synthesis. The transcriptional and post-transcriptional mechanisms and metabolic complexes regulating phenylpropanoid and lignin biosynthesis are still only partially known (Boerjan et al. 2003). Most of our current knowledge of regulatory mechanisms is based on analyses at the single gene level (Capellades et al. 1996, Hu et al. 1998, Gray-Mitsumune et al. 1999, Chen et al. 2000, Lauvergeat et al. 2002). The exploitation of genome-wide gene expression analyses such as microarrays have, however, started to extend our knowledge on the regulation of lignin biosynthesis (Kirst et al. 2004) and, more generally, on the potential connections between the DNA sequence and phenotypic variation (global molecular phenotyping) (Rockman & Kruglyak 2006).

In situ hybridisation (Hawkins et al. 2003), promoter deletion (Leyva et al. 1992, Hauffe et al. 1993, Feuillet et al. 1995, Lacombe et al. 2000, Lauvergeat et al. 2002) and microarray (Kirst et al. 2004) approaches have been used to study the regulation of lignin biosynthesis genes. It has been proposed that conserved AC-rich regulatory elements in the promoter of lignin genes direct the expression of e.g. *CCR* (Lacombe et al. 2000), *4CL* (Hauffe et al. 1993) and *CAD* (Feuillet et al. 1995) in vascular tissues. AC regulatory elements seem to contain both positive and negative cis-acting motifs which, in concert (Leyva et al. 1992, Hauffe et al. 1993, Lacombe et al. 2000) and potentially with the regulatory sequences scattered in the transcribed region of the gene (Soltani et al. 2006), orientate expression in the xylem and suppress it in other cells/tissues such as the cortical cells or phloem. In the genome-wide analysis of the lignification toolbox in *Arabidopsis thaliana*, AC regulatory elements were

found to be specific for the promoters of genes involved in G lignin synthesis (Raes et al. 2003). In addition to the cis-acting elements, the MYB (Tamagnone et al. 1998, Patzlaff et al. 2003, Karpinska et al. 2004, Newman et al. 2004, Goicoechea et al. 2005) and KNOX (Mele et al. 2003, Groover et al. 2006) families of transcription factors have been identified as regulatory elements of lignin genes. Furthermore, quantitative trait locus (QTL) analysis of the transcript levels (microarrays) of lignin-related genes in fast-growing *Eucalyptus* indicate that their mRNA abundance is regulated by two genetic loci, demonstrating coordinated genetic control over lignin biosynthesis and growth (Kirst et al. 2004).

GUS reporter gene fusions have been commonly used to investigate both spatial and temporal expression patterns directed by lignin gene promoters at the tissue level (Boerjan et al. 2003). Numerous studies have been carried out on both herbaceous model species and woody dicots showing that the expression of lignin-involved genes, e.g. *PAL* (Gray-Mitsumune et al. 1999), *C4H* (Zhao et al. 2005), *4CL* (Hu et al. 1998), *CCoAOMT* (Chen et al. 2000), *COMT* (Capellades et al. 1996) and *CAD* (Lauvergeat et al. 2002), are directed to lignifying tissues during plant development and as a response to non-developmental signals such as pathogen attack and wounding (Table 1, Boerjan et al. 2003).

In the work of Capellades et al. (1996), the *Zea mays* COMT promoter was able to direct GUS expression to lignifying cells and was induced by wounding and/or a fungal elicitor from *Phytophtora cryptogea* in transgenic *Zea mays* and *Nicotiana tabacum* L. The essential promoter signals inducing the specific COMT expression thus seem to be conserved in two evolutionary distinct and anatomically different species. The *in situ* analysis of *N. tabacum* L. showed a constitutive expression of COMT I in lignified tissues of different organs, i.e. the vascular tissues of stems and leaves, as well as an induced expression in the epidermis cells of leaves infected with tobacco mosaic virus (TMV) (Jaeck et al. 1996). In *in situ* analyses of the differentiating stem xylem of *Eucalyptus gunnii* Hook, on the other hand, the expression pattern of *COMT* was similar to that of *CAD* (Hawkins et al. 2003). In young stem internodes, *COMT* and *CAD* transcripts were associated with cells poor in S-type lignin (primary xylem vessels and immature secondary xylem cells), whereas in older stem internodes gene expression was evident in cells that had become rich in S-type lignin (mature secondary xylem cells). The coordinated expression of *COMT* and the majority of other lignin biosynthesis genes was found in the microarray analysis of *Eucalyptus* (Kirst et al. 2004). Furthermore, there was strong correlation between the expression of Cald5H and COMT, two adjacent enzymes involved in S unit synthesis (Kirst et al. 2004).

Genetic transformation of trees

General aspects

The transformation protocols based on *Agrobacterium*-mediated and/or direct gene transfer by biolistic bombardment have been successfully applied for the genetic transformation of numerous woody angiosperm species (Merkle & Nairn 2005), including *Populus* and *Betula.* The introduction of transgenes has been included in both sense and antisense strategies (referring to the orientation of the introduced gene into the plant genome) (Strauss et al. 1995, Baucher et al. 1998) and RNAi technology (Merkle & Nairn 2005). In the antisense strategy, duplex formation between the antisense transgene and the endogenous gene transcripts is proposed to induce the degradation of duplexes and, correspondingly, lead to suppressed gene expression (Strauss et al. 1995). The sense strategy was originally targeted for over-expression of the gene but, as originally observed through the introduction of chalcone synthase transgene into

Table 1. Analyses used in investigating tissue-specific, lignin-promoter-GUS activities involved in developmental and/or induced lignification in deciduous species.

Promoter	Target species for the GUS analysis	Developmental/induced promoter activity	Tissue specificity of promoter activity	Reference
Populus trichocarpa x P. deltoides PAL2	*P. trichocarpa x P. deltoides*	Developmental, and potentially associated with the synthesis of non-lignin related phenylpropanoids	Primary xylem and epidermal/ subepidermal cells of young tissues	Gray-Mitsumune et al. 1999
	Nicotiana tabacum	Developmental	Primary and/or secondary xylem	
Populus tomentosa C4H	*N. tabacum*	Developmental, and induced by wounding	Mainly in lignified tissues, i.e. xylem of vascular system in stems and midribs	Zhao et al. 2005
Populus tremuloides Michx.	*N. tabacum* Hanana			Hu et al. 1998
Pt4CL1		Developmental	Developing xylem	
Pt4CL2		Associated with the synthesis of non-lignin related phenylpropanoids	Epidermal cells	
P. trichocarpa gPtCCoAOMT1 gPtCCoAOMT2	*Populus tremula x P. alba*	Developmental, and induced by wounding, pathogen attack and mechanical bending	Lignifying cells and associated cells; cell specificity lost under mechanical bending	Chen et al. 2000
Eucalyptus gunnii CAD2	*P. tremula x P. alba*; *Vitis vinifera* L. *N. tabacum* L.	Developmental, and induced by wounding in *P. tremula x P. alba* and *N. tabacum*	Vascular tissues	Lauvergeat et al. 2002
		Associated with the synthesis of non-lignin related phenylpropanoids	Periderm of *V. vinifera,* root tips of *N. tabacum*	

petunia (Napoli et al. 1990), the sense strategy may also lead to silencing (down-regulation) of both the endogene and the transgene due to co-suppression (i.e. post-transcriptional gene silencing, PTGS). The molecular mechanism of the gene silencing was for long unclear until the relatively recent discovery of RNA interference (RNAi) (Yu & Kumar 2003, Matthew 2004, Chen 2005, Bonnet et al. 2006, Zhang et al. 2006). In the RNAi silencing process, the transgene gives rise to long double-stranded (ds) RNA molecules, which are enzymatically cleaved into very small pieces of RNA (c. 21 nt), referred to as small interfering RNAs (siRNAs). siRNAs are then incorporated in an RNA silencing system (RISC) which is able to recognize, bind and induce cleavage or translation repression of complementary mRNAs (Bonnet et al. 2006, Zhang et al. 2006). The RNAi technique is currently being applied for the efficient production of down-regulated or knock-out plants (Wesley et al. 2001), e.g. in genetic transformation of *Betula pendula* for achieving sterility (Lännenpää 2005).

Lignin-modified trees

The analysis of genetically modified or mutant plants with altered expression of lignin biosynthesis genes has proved to be an important tool in advancing our understanding of the complex emergence of the lignin-including monolignol biosynthesis pathway, polymer structure and the functional properties of lignin (reviewed by Boerjan et al. 2003, Hoffmann et al. 2004, Li et al. 2006). To date, transgenic plants or mutants with modified expression of most of the monolignol biosynthesis genes have been studied in detail (Boerjan et al. 2003). Considering the great economical and environmental impact of wood lignification on the pulp and paper industry, lignin properties that improve solubilisation would be highly beneficial on a global scale (Baucher et al. 2003). This can be achieved either by decreasing the amount of lignin and/or by increasing lignin solubility through modifications to its chemical composition. Laboratory-scale pulping experiments have provided important insights into the pulping characteristics of lignin-modified trees (Lapierre et al. 1999, Jouanin et al. 2000, Pilate et al. 2002, Huntley et al. 2003).

A reduced lignin content has been associated with down-regulation of several of the genes involved in lignin biosynthesis (reviewed by Boerjan et al. 2003). In woody angiosperms, a reduced lignin content has mostly been associated with the down-regulation of 4CL (Hu et al. 1999, Li et al. 2003), CCoAOMT (Meyermans et al. 2000, Zhong et al. 2000), and CAD (Lapierre et al. 1999, Pilate et al. 2002). Suppression of the *4CL* gene by antisense inhibition in *P. tremuloides* resulted in a significant decrease in the lignin quantity, which, in turn, was compensated by an increased cellulose content in the wood (Hu et al. 1999, Li et al. 2003). As one potential consequence of the enhanced deposition of cellulose, transgenic trees were able to sustain structural integrity despite the significant loss of lignin in their woody xylem (Hu et al. 1999). Down-regulation of CCoAOMT by the antisense/sense approach in *Populus* was associated with a decrease in both S and G units and thus in the total quantity of lignin (Meyermans et al. 2000, Zhong et al. 2000). The lowered lignin quantity did not affect plant growth, but was potentially compromised by the presence of deformed vessel elements in transgenic plants (Zhong et al. 2000). The down-regulation of CAD in Populus trees was associated with both a slight decrease in the lignin quantity and changes in lignin structure. The increased frequency of free phenolic groups in β-O-4 linked G or S units improved lignin solubilisation and fragmentation during kraft pulping (Lapierre et al. 1999, Pilate et al. 2002), thus decreasing the consumption of pulping chemicals and improving the cellulose quality (degree of polymerization = DP) due to the milder pulping requirements (Pilate et al. 2002).

The S units are more amenable to chemical dissolution due to their ability to form both frequent and labile β-O-4 interunit bonds compared to the G units involved in the stable carbon-carbon linkages (Baucher et al. 1998). As a consequence, the degree of lignification in wood pulping is directly proportional to the ratio of the S to G contents (Chang & Sarkanen 1973, Chiang & Funaoka 1990). Based on wood-pulping kinetics, it has been estimated that one unit increase in the lignin S/G ratio would approximately double the rate of lignin removal (Chang & Sarkanen 1973). This has raised wide interest in the enrichment of S units in transgenic plants by genetic modifications of *F5H/Cald5H* (Franke et al. 2000, Huntley et al. 2003, Li et al. 2003) and *COMT/AldOMT* (Tsai et al. 1998, Lapierre et al. 1999, Jouanin et al. 2000).

Over-expression of the *F5H/Cald5H* gene under the control of lignification-associated promoters has resulted in lignin with highly enriched S units in *Arabidopsis* (Meyer et al. 1998), *N. tabacum, P. tremula x P. alba* (Franke et al. 2000, Huntley et al. 2003) and *P. tremuloides* (Li et al. 2003), thus supporting the role of F5H/Cald5H as an entry point for S lignin synthesis. The increased S/G ratio in the lignin of C4H-F5H-transformed trees was also associated with improved pulping efficiency, as determined by the lower residual lignin content of the pulp, improved delignification under milder processing conditions, beneficial effect on the cellulose content and quality (degree of polymerization) of the pulp and a reduced need for bleaching and a reduced chemical load (Huntley et al. 2003). The over-expression of F5H may thus offer a potential option for the genetic modification of the S/G composition in the woody angiosperms used by the pulp and paper industry.

The implementation of COMT modifications in *Populus* species by modifying the COMT activity using the sense and/or antisense strategy have mainly resulted in suppressed COMT activity with a concomitant reduction in S unit content and the incorporation of novel monolignol, 5-hydroxyconiferyl alcohol (5-OH-G), into lignins (Van Doorsselaere et al. 1995, Tsai et al. 1998, Lapierre et al. 1999, Jouanin et al. 2000, Pilate et al. 2002). When COMT is down-regulated, CAD can apparently catalyze the reduction of 5-hydroxyconiferyl aldehyde intermediates to 5-OH-G units (Ralph et al. 2001a, Ralph et al. 2001b), which are readily incorporated into lignin through the production of cyclic benzodioxane (4-O-β/5-O-α) structures (Ralph et al. 2001a, Ralph et al. 2001b, Morreel et al. 2004, Ralph et al. 2004). These 5-OH-G derived benzodioxane units have been found to be the second most abundant interunit type in the *COMT*-gene-silenced *P. tremula x P. alba*, suggesting that they may quantitatively compensate for the sinapyl alcohol deficiency (Ralph et al. 2001a, Ralph et al. 2001b). Unlike S units, benzodioxanes are inefficiently degraded under alkaline pulping conditions and their presence thus lowers the pulping efficiency of COMT-deficient plants (Ralph et al. 2001b, Ralph et al. 2004). The profound changes in lignin composition resulting from the suppressed COMT activity show that COMT is essential for the production of S unit precursors. On the other hand, the increased proportion of G units, together with the increased proportion of resistant interunit linkages, made the COMT-modified wood less amenable to laboratory-scale delignification (Lapierre et al. 1999, Jouanin et al. 2000, Pilate et al. 2002).

Ecological interactions of genetically modified (GM) trees

Forest trees hold a key position in forest ecosystems due to their longevity and numerous biotic interactions with other organisms in the ecosystem. Public concern has raised the need for scientific evaluation of the potential environmental benefits/disadvantages associated with genetically modified (GM) trees (Merkle & Nairn 2005, Farnum et al. 2007, Gartland & Oliver 2007). The potential subjects of concern include gene flow, i.e. dispersal of pollen, seeds

or vegetative propagules between the GM tree cultivars and natural forests, the subsequent interaction between gene flow and genetic drift (natural selection) and the resulting differences in fitness introduced by transgenes (Farnum et al. 2007), potential horizontal gene transfer (HGT), e.g. from decomposing transgenic plant material to soil microbes, transgene stability in field conditions and/or over the long rotation times of trees, and unintended effects of GM trees on non-target species (Finstad et al. 2007). In order to counteract these risks, tools have been developed to control transgene spread, e.g. by preventing flowering or vegetative reproduction (root suckers) (Brunner et al. 2007, Farnum et al. 2007). In order to evaluate the safety and approval of GM trees prior to field testing and potential commercial plantings, the USA, Canada and European Union have enacted stringent regulations for the testing and release of GM products (Nehra et al. 2005, Finstad et al. 2007, Sederoff 2007). The most important features of EU legislation include openness (public hearings in the application phase and a public register) and careful risk assessment that also includes the potential long-term and unforeseeable environmental effects (Council Directive 90/219/EEC, Directive 2001/18/EC of the European Parliament, Geenitekniikkalaki (377/1995), Valtioneuvoston asetus geenitekniikasta (928/2004)). Improved public acceptance is necessary for the wider application of GM forest trees in commercial forestry. Another requirement is related to forest certification, because the current systems, e.g. that of the Forest Stewardship Council (FSC), do not allow the cultivation of GM trees at all (Gartland & Oliver 2007).

In respect to the numerous functions of lignin, there are various ways in which modified lignin might affect biological interactions (reviewed by Halpin et al. 2007). The changed secondary cell wall structure resulting from lignin modification may affect the feeding behaviour and/or growth performance of leaf-feeding and stem-boring herbivores. Lignin modification may have an impact on defence responses against pathogens or, when related to the chemical composition of the roots, to the ability of trees to form symbiotic interaction with mycorrhizal fungi. Being a hard-to-decay component, lignin modification could also exert effects on plant decomposition and, consequently, affect the interactions with soil organisms and nutrient transformations (Halpin et al. 2007).

Our current understanding of the potential ecological effects of lignin-modified trees is limited (Pilate et al. 2002, Tilston et al. 2004, Halpin et al. 2007). The profiles of CAD- and COMT-down-regulated *P. tremula x P. alba* trees did not differ as regards the visiting/resident insects, leaf-damage caused by them, damage caused by fungi and bacteria, or microbial diversity in the soil, compared to non-modified controls when grown for 4 years in the field (Pilate et al. 2002, Halpin et al. 2007). The rates of trunk decomposition or production of soil microbial biomass of the transgenic trees were not affected by the CAD- or COMT-down-regulation (Tilston et al. 2004), but the early decomposition rate of the roots of the transgenic lines was slightly higher than that of the controls (Pilate et al. 2002). Short-term decomposition studies on CAD- and COMT-modified tobacco plants under laboratory conditions supported the latter observation (Webster et al. 2005, Hénault et al. 2006, Hopkins et al. 2006), and it was proposed that the modified lignin may have contributed to the decreased protection of labile polysaccharide components in the cell walls during early decomposition (Pilate et al. 2002, Webster et al. 2005, Hénault et al. 2006). Furthermore, the residues of CAD- and COMT-down-regulated tobacco plants were preferentially decomposed by fungi over Gram-positive bacteria, indicating possible changes in the composition of the soil microbial community (Hénault et al. 2006). In field conditions, on the other hand, where the effect of lignin modification may be less significant than that of environmental variability on the trees (Pilate et al. 2002), the potential ecological effects of lignin-modified trees needs to be verified in long-term studies and in relation to the normal range of variation of the biological traits.

AIMS OF THE THESIS

Lignin is one of the main structural elements of wood, and in angiosperms it is mainly composed of G (guaiacyl) and S (syringyl) monomers (Baucher et al. 1998, Boerjan et al. 2003). Considerable scientific interest has focused on the formation of S lignin in woody angiosperms due to the association between the wood S lignin content and the delignification properties i.e. in chemical pulping processes (Baucher et al. 2003). COMT (caffeate/5-hydroxyferulate *O*-methyltransferase) is one of the key enzymes specific to the formation of S lignin. Most of our current understanding on the role of lignin biosynthesis genes, including *COMT*, is based on the evidence gained from the analysis of natural lignin mutants or genetically modified plants (Boerjan et al. 2003, Li et al. 2006). Despite the great scientific progress made in the field, our knowledge of the regulation mechanisms of lignin biosynthesis, for instance, is still inadequate (Li et al. 2006). Furthermore, comprehensive investigations of the potential ecological effects of lignin-modified trees are clearly needed for evidence-based decision-making (Halpin et al. 2007).

Silver birch (*Betula pendula* Roth) is economically the most important deciduous tree species in the Nordic countries as it provides raw material e.g. for the chemical pulp industry. In Finland, silver birch is the most important broad-leaf species of conventional tree breeding (Koski & Rousi 2005), and it is also suitable for molecular breeding due to the existence of micropropagation methods (Ryynänen & Ryynänen 1986), genetic transformation techniques (Keinonen-Mettälä et al. 1998, Valjakka et al. 2000) and EST libraries (Palva 2000). In Nordic forest ecosystems, silver birch is one of the key species with widely characterised ecological interactions (Rousi et al. 1997, Laitinen et al. 2004, Koski & Rousi 2005).

The main aim of the present study was to investigate lignin biosynthesis by focusing on the effects and regulation of the *COMT* gene in silver birch. This was carried out with the help of genetically transformed PtCOMT lines and by using lines with PtCOMT-promoter-GUS constructs, respectively. Moreover, the interactions between the PtCOMT-modified *B. pendula* and leaf-feeding herbivores/ectomycorrhizal fungus were analysed *in vitro*.

The specific aims of the studies were:

1) To examine the effect of the sense-*PtCOMT* gene transfer on lignin biosynthesis in *B. pendula* stem wood, leaves and roots (**I, III, IV**)

2) To investigate the spatio-temporal expression pattern of 900-bp and partly deleted PtCOMT promoters in *B. pendula* during the course of the growing season and under mechanical bending and wounding (**II**)

3) To examine the potential effect of PtCOMT modification on the feeding performance and relative growth rate of common leaf-feeding insect herbivores of *B. pendula in vitro* (**III**)

4) To analyse the potential effect of the PtCOMT modification on the ectomycorrhizal interaction between *B. pendula* and *Paxillus involutus*, and the resulting growth responses of *B. pendula in vitro* (**IV**)

5) To review the current state of the production and potential environmental effects of the lignin-modified trees (**V**)

MATERIAL AND METHODS

Production of transgenic *Betula pendula* lines

Two gene constructs containing the caffeate/5-hydroxyferulate *O*-methyltransferase (*COMT*) gene from *Populus tremuloides* (L.) (Gene Accession number U13171) under the control of cauliflower mosaic virus (CaMV) 35S promoter or sunflower polyubiquitin (UbB1) promoter were used for the genetic transformation of *in vitro* shoot cultures of *B. pendula* clones E5396, 98, A and R (**I, III-V**). For the PtCOMT-promoter analyses (**II**), two gene constructs containing *GUS* (β-glucuronidase = *uid*A) reporter gene driven either by 900-bp (signed as p including nucleotides -1 to -886) or partly deleted (signed as dp including nucleotides -1 to -414) PtCOMT promoter were used for the transformation of *B. pendula* clone A. All the transgene constructs contained neomycin phosphotransferase II (*nptII*) gene driven by CaMV 35S promoter as a selective marker gene. All the transformations were performed by microprojectile bombardment using internode sections of *in vitro* shoots as targets. The transformed material was cultivated on Woody Plant Medium (WPM) (Lloyd & McCown 1980) with the kanamycin selection, and callus-forming tissues were subjected to shoot induction, as described in the original articles (**I-III**). One individual shoot per callus was selected for multiplication to start a specific genetically modified line. The rooted shoots were potted and transferred to a greenhouse.

Greenhouse cultivation of the transgenic birches (I-III)

The transgenic silver birch plants and non-transgenic controls were cultivated under the standard greenhouse conditions as described in the original articles (**I-III**). The growing season followed the natural growth period, starting approximately at the beginning of May and lasting till September. The plants were organized in randomly assigned blocks and surrounded by extra birches of the same lines in order to stabilise the growth conditions. The transgenic plants and controls were cultivated under the same conditions to be used for DNA and RNA isolations. The stem height and stem diameter growth were measured each growing season and the overall morphology and growth habit of the plants were studied visually during the growing season (**I**). The plants following the normal (i.e. not belated) growth rhythm were decapitated after the 2nd growing season because of the limited greenhouse space (**III**).

DNA and RNA analyses

Total genomic DNA was isolated from the leaves, stems and roots by the modified method of Lodhi et al. (1994), as described in Valjakka et al. (2000) (**I-III**). Southern blot analyses were performed to verify the integration of the transgenes into the birch genome. For the hybridisations, digoxigenin-11-dUTP-labeled double stranded probes were used according to the conditions described in the original articles (**I-III**). The chemiluminescence detection of hybridisation products was performed according to the manufacturer's (Roche Diagnostics GmbH) instructions.

Total RNA was isolated from leaves, developing xylem and phloem by the modified method of Chang et al. (1993) during the growing season. Northern blot analyses were performed to study the functioning of the transgenes in the birch tissues. Double stranded, digoxigenin-11-dUTP-labeled DNA probes were used for the hybridisation as described in the original experiments (**I-III**). The expression of endogenous birch *COMT* was analysed from RNA

samples with a probe based on the sequence information from a cluster of expressed sequence tags (ESTs) representing birch *COMT*s in developing xylem (provided by Dr. Kauppinen and Prof. Helariutta, University of Helsinki, Finland) (**I**). The hybridisation products were detected according to Roche Diagnostics GmbH´s instructions.

Lignin analysis

For the lignin analyses, stems, leaves and roots were first extracted with benzene/acetone-ethanol or acetone-ethanol-water (**I, III, IV**). The Klason lignin (acid-insoluble lignin) content of the extractive-free material was analysed gravimetrically by the modified method of Effland (1977). The acid-soluble lignin was determined by UV absorption at 203 nm on a spectrophotometer using a lignin absorptivity of 110 l g^{-1} cm^{-1}. The syringyl:guaiacyl (S/G) ratio of the lignin was determined by a modified thioacidolysis method (Rolando et al. 1992), which is based on the solubilisation of lignin via the breakage of β-O-4 interunit linkages and subsequent gas-chromatography-mass spectral characterizations of the liberated monomeric lignin units.

Histochemical GUS assay and microscopical analysis of transgenic plants with PtCOMTp- or PtCOMTdp-promoters (II)

The functioning of the PtCOMT promoters in PtCOMTp- and PtCOMTdp-GUS birches was investigated by the histochemical GUS enzyme activity assay as described by Jefferson (1987) and modified by Aronen et al. (1994). The preliminary screening of the GUS expression was performed on the hand-cut sections, whereas for the detailed analysis of the spatio-temporal expression patterns, as well as of the expression patterns resulting from stem bending, microtome-cut thin sections were used. The microscopical analyses based on the bright field and fluorescence assays were performed according to Rech et al. (2003).

The bending and wounding experiments of transgenic plants with PtCOMTp- or PtCOMTdp-promoters (II)

The potential effects of stem bending and wounding on PtCOMTp- and PtCOMTdp-promoter activity were investigated during active growth in the growing season. In the bending experiment, the uppermost parts of the stems were bent with wire for 4 weeks, whereafter samples were taken from non-bent, bent and re-orientated stem parts for microscopical analysis of the GUS expression. In the wounding experiment, the stems were wounded by cutting with a knife, and the GUS expression was followed histochemically on stems prepared for hand-cut sections during the one-week period after wounding.

Feeding experiments with insect herbivores (III)

The insect herbivores used in the feeding experiments were common, polyphagous birch-feeding species including the moths *Aethalura punctulata* (Denis & Schiffermüller), *Cleora cinctaria* (Denis & Schiffermüller) and *Trichopteryx carpinata* (Borkhausen) (Lepidoptera: Geometridae), and the leaf beetles *Agelastica alni* (L.) (Coleoptera: Chrysomelidae) and *Phyllobius* spp. (Coleoptera: Curculionidae). The moths and adult leaf beetles were collected at the time of their occurrence in spring/early summer. For the production of larvae, one female moth with one male was left to lay eggs on birch twigs in a growing chamber and,

when hatched, the larvae were left to develop until they reached the size of 1-1.5 cm. Fully opened, even-aged leaves including the petioles were used in the feeding experiments as described in the original article (**III**).

The food selection experiment was carried out as a two-choice experiment by letting the herbivore feed in a Petri dish containing one transgenic and one control (the same birch genotype without gene transferring) leaf. At the end of the experiment, the leaf consumption was determined as the difference between the initial and final leaf area, and the more consumed leaf was registered as the selected one. In the relative growth rate (RGR) experiment, the weight of the larva was first determined, whereupon it was put in a Petri dish containing one transgenic or one control leaf. At the end of the RGR experiment, the final weight of the larva was determined, and the RGR of the larva was calculated as the proportional weight gain during the experiment. In both the food selection and RGR experiments, leaf vein consumption was also calculated as the percentage of consumed mid-vein and principal lateral veins per leaf.

Inoculation of *B. pendula in vitro* plants with *Paxillus involutus* (IV)

The inoculation experiment with the ECM fungus *Paxillus involutus* (Batsch) Fr. was performed with PtCOMT plants *in vitro*. The *in vitro* birch plantlets were inoculated individually by placing mycelial agar plugs close to the root system. In the non-inoculated control cultures, fungal mycelium was substituted by sterile agar plugs. The root-plug-system was covered, protected from light, and the dishes were placed in tracks at an angle of 70° in the culture room for 8 weeks. At the time of harvest, the number of viable plants, the number of adventitious and lateral roots, the dry mass of the shoots and roots and the number of lateral roots covered with the fungal hyphae, were determined. Samples of roots with the fungal hyphae were taken for examination by light microscopy.

Statistical analysis

The data were analysed using the SPSS (**I, III-IV**, SPSS Inc., Chicago, IL, USA) 10.0-13.0 statistical software. Statistical significant differences in growth among the transgenic and control birch lines/clones were determined by analysis of variance or by non-parametric analysis of variance (Kruskal-Wallis test), followed by pairwise comparisons using Tukey's HSD test or the Mann-Whitney test (at the 5% risk level) (I).

In the feeding experiments (**III**), the data were transformed when necessary to meet the assumptions of valid statistical tests. The leaf lignin results were analysed with using analysis of variance and pairwise comparisons performed with Tukey's HSD test Tamhanes's test. For the analysis of the food selection experiments, leaf consumption was converted into a binary response (preferred or not preferred) and, in the case of homogeneity (according to the test of heterogeneity), the transgenic leaf vs. control leaf selection was analysed with the χ^2-test in the pooled data. Otherwise the comparisons were performed between the individual transgenic and control line. The RGR values were scaled between 0-1 and statistical analyses were performed with analysis of covariance, followed by the Šidák´s *t*-test for pairwise comparisons of the estimated marginal means. The leaf vein consumption data were analysed either with analysis of variance or with non-parametric Kruskal-Wallis test, and pairwise comparisons were performed using the Dunnett's or Tukey's HSD test (**III**).

The data obtained from the inoculation experiments (IV) were transformed, when necessary, and analysed with a parametric t-test, non-parametric Mann-Whitney U-test or the Kruskall-Wallis-test combined with the Mann-Whitney U test with a Bonferroni correction.

RESULTS

Production of transgenic *B. pendula* lines

The *PtCOMT* gene was successfully introduced into *B. pendula*, and two 35S-PtCOMT lines (23 and 44) and two UbB1-PtCOMT lines (65 and 130) representing clone A (**I, III-V**) were regenerated, multiplied and grown in a greenhouse together with non-modified controls. In addition, one UbB1-PtCOMT line (110) representing clone E5396 (**IV**) was regenerated, multiplied and grown *in vitro* together with the non-modified control clone. In the PtCOMT-promoter-experiment (**II**), PtCOMTp-GUS and PtCOMTdp-GUS constructs were successfully introduced into clone A, and six and eleven transgenic lines respectively were produced and grown in a greenhouse together with non-modified controls. From the PtCOMT-promoter lines, two PtCOMTp-GUS and seven PtCOMTdp-GUS lines were selected for the Southern and Northern blot analyses based on the positive results of the histochemical GUS assay in preliminary screening and good *in vitro* multiplication characteristics.

Southern and Northern blot analyses of transgenic *B. pendula* lines

Southern hybridisation analysis confirmed the stable integration of 35S-*PtCOMT*, UbB1-*PtCOMT*, PtCOMTp-*GUS*, PtCOMTdp-*GUS* and *nptII* transgenes in the genome of the transgenic lines. In transgenic lines 23, 44, 65, 130 and 110 there were up to five copies of the *PtCOMT* gene (**I, III-IV**). The copynumber of PtCOMTp-*GUS* and PtCOMTdp-*GUS* transgenes varied from one to six in individual transgenic lines (**II**).

The Northern hybridisation analysis verified the expression of the *PtCOMT* gene in the leaves, phloem and developing xylem of transgenic lines 23, 44 and 130. In line 65, the expression of the *PtCOMT* was detected in the leaves and phloem. In all the samples, the size of the UbB1-PtCOMT transcript was larger than that of the 35S-PtCOMT transcript. The expression of the molecular marker gene *nptII* was found in all the samples of lines 23, 44, 65 and 130 (**I, III**). When the probe based on EST sequence data of silver birch *COMT* data (kindly provided by Dr. Kauppinen and Prof. Y. Helariutta, University of Helsinki) was used, it recognized a signal of approximately the same size as the *PtCOMT* mRNA in leaf, phloem, and xylem samples of both the control and the UbB1-PtCOMT lines (**I**). The hybridisation analyses were not performed for line 110. The expression of PtCOMTp-*GUS*, PtCOMTdp-*GUS* and *nptII* genes was established in xylem isolated from the uppermost part of the stems and stem base (**II**).

Greenhouse observations

Stem height and diameter growth and morphological characteristics of lines 23, 44 and 65 were similar to those of the non-modified control plants during the two-year greenhouse experiment. Line 130 showed a dwarf phenotype in the first growing season. During the second growing season, the onset of growth of line 130 was delayed, but during the course of the season its growth was accelerated and only a small but significant difference in stem height growth was found compared with the control and other lines. Line 130 also had a differing growth habit, with no branching and dark green, warped leaves (**I**). Excluding the PtCOMTp-GUS line 28, the growth and morphology of the transgenic lines used for the PtCOMT promoter experiments (**II**) were similar to those of the non-modified control clone. Line 28 was characterised by a shorter stem and warped leaves compared to the control clone.

Lignin analysis

The Klason lignin content of the transgenic lines did not differ significantly from the non-modified control clone, except in the case of line 130 (Table 2). In this line, the two-year-old stems contained less Klason lignin than those in control clone A, while in the three-year-old stems, however, the Klason lignin contents of line 130 and control clone A did not differ (**I**). In the roots of line 130, the Klason lignin content was higher than in the non-modified control clone, but there was no significant difference in the total lignin content (Table 2).

The S/G ratio of the lignin in the leaves, stems and roots of the two 35S-PtCOMT lines (23 and 44) was lower than that of control clone A (Table 3). In addition, abnormal 5-OH G units were present in the stem wood lignin of both 35S-PtCOMT lines (**I**). In the UbB1-PtCOMT lines 65, 130 and 110 there were no marked differences in the S/G ratios compared to the control clones (Table 3). Inoculation with the ectomycorrhiza (*Paxillus involutus*) had no consistent effect on the S/G ratios of the *in vitro* roots (Table 3).

Histochemical GUS assay and microscopical analysis of transgenic plants with PtCOMTp- or PtCOMTdp-promoters (II)

Preliminary screening of GUS expression

In the preliminary screening of the PtCOMTp-GUS and PtCOMTdp-GUS lines, GUS expression was found in leaves from the 4th to the 12th week and in stem and/or branch pieces from the 4th or 6th to the 12th week of the first and/or second growing season. In the leaves, GUS expression was most evident in the hair cells and leaf veins, whereas in the leaf mesophyll the GUS expression was occasional and faint.

Temporal and spatial GUS expression

In the base and uppermost part of stems, the PtCOMTp-GUS expression was mainly seen in the cells of new xylem (fibres, vessels and ray cells) and occasionally in sieve tube cells from the 8th to 13th week during the growing season. The GUS expressing new xylem was clearly distinctive from that non-expressing old xylem. The GUS expression was not observed from the 18th week on during the growing season. In the PtCOMTdp-GUS line, the GUS expression patterns were in most cases similar to those of the PtCOMTp-GUS line. In addition, stem hair cells, some old xylem cells and pith cells occasionally showed PtCOMTdp-GUS expression. In the uppermost part of the stems, a faint and overall PtCOMTdp-GUS expression was also observed on the 18th and 26th week during the growing season.

In the roots, the PtCOMTp-GUS and PtCOMTdp-GUS expression mainly followed the same spatio-temporal pattern as that seen in the stem wood. The most uniform/strongest GUS expression was, however, seen in new xylem on the 13th week during the growing season and thus later than that in the stem bases (**II**).

GUS expression in the transgenic plants with PtCOMTp- or PtCOMTdp-promoters induced by stem bending and wounding

In the PtCOMTp- and PtCOMTdp-lines, a strong GUS expression was induced in the phloem sieve tubes and new and old xylem in tension wood formed as a result of stem bending. The wounding did not simulate GUS expression in the stems of PtCOMTp- or PtCOMTdp-lines (**II**).

Table 2. The Klason, acid-soluble and total lignin (mean ± SE) of the leaves, roots and/or stems of the PtCOMT-modified *B. pendula* lines and control clones. Statistically significant differences between the means ($P < 0.05$) are marked with different letters.

Clone/Line	Klason lignin %, mean ± SE			Acid-soluble lignin %, mean ± SE		Total lignin %, mean ± SE	
	leaves (**III**)	stem (**III**)	root	leaves (**III**)	root	leaves (**III**)	root
clone A	15.0 ± 0.6^{ab}	24.7 ± 0.3^{ab}	14.6 ± 0.2^{a}	3.2 ± 0.1^{a}	3.0 ± 0.0^{a}	18.2 ± 0.6^{ab}	17.6 ± 0.2^{a,b}
23	17.3 ± 0.7^{a}	23.5 ± 0.4^{a}	14.5 ± 0.3^{a}	3.6 ± 0.1^{b}	1.7 ± 0.1^{b}	21.0 ± 0.7^{a}	16.2 ± 0.4^{a}
44	15.6 ± 0.4^{a}	23.3 ± 0.5^{a}	15.3 ± 0.2^{a,b}	3.0 ± 0.2^{ac}	1.6 ± 0.0^{b}	18.7 ± 0.4^{ab}	16.9 ± 0.2^{a}
65	16.5 ± 0.2^{a}	26.1 ± 0.3^{b}	14.3 ± 0.4^{a}	2.7 ± 0.1^{c}	2.9 ± 0.0^{a}	19.2 ± 0.2^{a}	17.1 ± 0.4^{a}
130	12.9 ± 0.2^{b}	17.7 ± 0.5^{c}	16.8 ± 0.3^{b}	4.1 ± 0.0^{d}	2.6 ± 0.0^{c}	17.0 ± 0.2^{b}	19.4 ± 0.3^{b}
clone E5396	-	-	14.2 ± 0.1^{a}	-	2.9 ± 0.0^{a}	-	17.1 ± 0.1^{a}
110	-	-	14.4 ± 0.9^{a}	-	2.7 ± 0.1^{a}	-	17.1 ± 0.9^{a}

- = Not determined.

Table 3. S/G ratios (mean ± SE) of lignin in the leaves, stems and roots of greenhouse-grown (GH) and/or PtCOMT/control birches cultivated *in vitro* in the absence or presence of ectomycorrhizal (- ECM/+ ECM) fungi. The results are based on 1-4 determinations, and statistical analyses were not performed.

Clone/Line	S/G mean ± SE				
	GH leaves (**III**)	GH stem (**III**)	GH roots	*in vitro* roots - ECM (**IV**)	*in vitro* roots + ECM (**IV**)
clone A	1.05 ± 0.04	2.27 ± 0.02	2.56 ± 0.09	0.85	0.72 ± 0.01
23	0.26 ± 0.06	0.63 ± 0.02	0.61 ± 0.06	0.41 ± 0.01	0.51 ± 0.07
44	0.66 ± 0.05	0.71 ± 0.03	0.86 ± 0.03	-	-
65	1.05 ± 0.04	2.14 ± 0.02	2.70 ± 0.25	0.66 ± 0.08	0.76 ± 0.06
130	1.03 ± 0.02	-	2.61 ± 0.12	0.80	0.62 ± 0.06
clone E5396	-	-	2.74 ± 0.01	0.64 ± 0.02	0.62 ± 0.03
110	-	-	2.29 ± 0.03	0.79	0.89 ± 0.08

- = Not determined

The effects of the PtCOMT modification on insect herbivores (III)

Two lepidopteran species, *A. punctulata* and *C. cinctaria*, preferred the leaves of line 130, and *A. punctulata* also leaves of line 23, over the control. *T. carpinata, A. alni* and *Phyllobius* spp. selected transgenic and control leaves equally. The relative growth rate (RGR) of the herbivores on the transgenic birch leaves did not differ compared to RGR on the control leaves. The RGR values of *A. punctulata* and *T. carpinat*a larvae varied according to the transgenic line. *A. punctulata* larvae grew better when they fed on leaves of line 130 than on

the leaves of lines 23 or 44. *T. carpinata* larvae showed the best RGR on leaves of line 65 and the lowest on leaves of line 23, although this difference was not statistically significant.

The leaf vein consumption indicated that geometrid larvae utilised more leaf veins than coleopteran species. Based on the food selection data, *Phyllobius* spp. consumed significantly more leaf veins of line 23 than the veins of the other lines. The leaf vein consumption of *A. alni* and lepidopterans was not significantly affected by the lignin modification (**III**).

The effects of the PtCOMT modification on the ectomycorrhizal interaction between *B. pendula* and *P. involutus in vitro* (IV)

The effects of P. involutus *on the viability, growth and root development of* B. pendula

The inoculation with *P. involutus* increased the percentage of viable *in vitro* plants, especially in transgenic lines 130 and 110. The inoculated plants of clones A and E5396 and transgenic lines 23 and 65 had a higher shoot and root dry weight compared to the non-inoculated plants. When the control clones were compared with their transgenic lines, the inoculated control clone E5396 had a significantly higher shoot dry weight than line 110.

The formation of adventitious roots was not affected by the inoculation, and it did not differ between the control clones and the corresponding transgenic line/s when inoculated with *P. involutus* or grown without the fungus. The inoculation increased the formation of lateral roots in control clone A and transgenic lines 23 and 65. When inoculated with *P. involutus*, control clone A formed less lateral roots than transgenic line 23 and more lateral roots than transgenic line 130. Transgenic line 110 was inferior in the formation of lateral roots to control clone E5396 both in the absence and in the presence of *P. involutus*.

Formation of ectomycorrhizas (ECMs)

Both control clones and transgenic lines were able to form ECMs. The characteristic of ECM, the percentage of lateral roots covered with the fungal hyphae, was higher in control clone A than in its transgenic line 65 and also slightly higher than in line 23. The formation of the Hartig net differed between control clone A and transgenic lines 23 and 130, and between control clone E5396 and transgenic line 110. In the roots of line 23 the fungal hyphae penetrated less frequently along the radial cell walls of the epidermis than in clone A. The percentage of lateral roots covered by the fungus was higher in line 130 than in clone A, but the fungus penetrated less deep along the radial cell walls of the epidermis. Line 110 was inferior to control clone E5396 in the formation of lateral roots covered by the fungal hyphae, and the penetration of fungal hyphae was reduced in line 110 compared to control clone E5396.

DISCUSSION

Production of PtCOMT-transgenic *B. pendula* lines

In the biolistic transformation of *B. pendula* with *PtCOMT* transgene, two 35S-PtCOMT lines (23 and 44) and two UbB1-PtCOMT lines (65 and 130) originating from clone A and one UbB1-PtCOMT line (110) originating from clone E5396 were produced. The Southern hybridisation verified the stable integration of transgenes into the plant genomes, and several copies of the transgenes were found in PtCOMT lines. In previous reports on deciduous tree species, particle bombardment has also resulted in multiple copies of inserted transgenes (Wilde et al. 1992, Serrano et al. 1996, Valjakka et al. 2000). The functioning of transgenes under the 35S- and UbB1-promoters was verified in the leaves, roots and developing xylem with Northern hybridisation. Northern hybridisation indicated that the UbB1-PtCOMT transcripts were larger than those of the 35S-PtCOMT signals, which may contribute to the different transcription starting site inherited in the two promoters. On the other hand, the insertion of transgenes into plant genomes may result in complex nucleotide rearrangements, i.e. deletions of the genomic sequence or the presence of filler DNA on insertion junctions, as shown in transgenic *Populus* trees (Kumar & Fladung 2002). Especially, the integration of a transgene into AT-rich genomic regions or regions with repeated DNA may lead to variable expression of the transgene (Kumar & Fladung 2001).

Lignin composition of PtCOMT-transgenic *B. pendula* lines

The *B. pendula* lines carrying the 35S-*PtCOMT* transgene had a decreased S/G ratio in the stem, leaf and root lignin, and abnormal 5-OH-G units were incorporated into the stem wood lignin. The amount of lignin in the 35S- or UbB1-PtCOMT lines did not differ from that of the unmodified *B. pendula* plants. The high sequence similarity between the endogenous and introduced *COMT* genes has apparently resulted in RNA interference based suppression of the *COMT* gene activities. The modified lignin composition caused by the 35S-*PtCOMT* transgene is in line with our current understanding of lignin biosynthesis in angiosperms and the essential role of COMT for formation of S unit (Van Doorsselaere et al. 1995, Tsai et al. 1998, Lapierre et al. 1999, Li et al. 2006). The lower S/G ratio of *in vitro* roots compared to the roots of greenhouse-grown plants (Table 3), found in the present study, most probably indicates later deposition of S units to G units in xylem differentiation, which has also been reported by Terashima et al. (1986).

Based on the delignification characteristics of transgenic *Populus* trees (Lapierre et al. 1999, Jouanin et al. 2000, Pilate et al. 2002, Baucher et al. 2003), the present *B. pendula* wood with a modified lignin composition can be expected to show decreased delignification in alkaline (kraft) pulping. The severely reduced S lignin content and frequency of labile βO-4 bonds in contrast to the enrichment of G units and resistant interunit bonds and decrease in free phenolic units (Lapierre et al. 1999, Jouanin et al. 2000, Pilate et al. 2002), are structural changes in COMT-suppressed wood associated with the decreased lignin solubility in alkaline cooking. In addition, the presence of 5-OH-G units may further deteriorate the wood pulping properties of COMT-suppressed wood because the 5-OH-G derived benzodioxane units are inefficiently degraded under kraft pulping conditions (Ralph et al. 2001b, Ralph et al. 2004). As a conclusion, the lignin composition of 35S-PtCOMT modified *B. pendula* lines support the current view on the decreased performance of COMT-suppressed wood for wood pulping

(Baucher et al. 2003), and the role of COMT as a key enzyme for S unit synthesis (Li et al. 2006).

Growth and morphological characteristics of the transgenic lines

The PtCOMT-transgenic lines with a decreased S/G ratio did not show altered growth or morphological characteristics under greenhouse conditions. These results are in line with the COMT-modified *Populus* plants grown in greenhouse and field conditions (Van Doorsselaere et al. 1995, Jouanin et al. 2000, Pilate et al. 2002). Compared to the control clone, UbB1-PtCOMT line 130 had a delayed growth rhythm and untypical morphology with a dwarfed phenotype, unbranched stems and altered leaf morphology. Presumably due to the delayed onset of growth, the lignin content of the stem wood of line 130 transiently decreased compared to that of the non-modified control clone. UbB1-PtCOMT line 110 grown *in vitro* and PtCOMTp-GUS transgenic line 28 grown in greenhouse conditions also had altered developmental characteristics compared to the control clone. These growth and morphological effects in the transgenic lines are likely due to the integration of a transgene into genomic sequences associated with normal plant growth and development, such as plant hormone metabolism and sensitivity. In transgenic *B. pendula* (Piispanen et al. 2003) and *Populus* (Han et al. 1997, Tzfira et al. 1999, Grünwald et al. 2001) trees, the altered *rol*-gene expression has been shown to be associated e.g. with reduced internodal stem length, abundant lateral branches and irregular leaf development, i.e. dark green, wrinkled and thick leaf appearance. The dwarfism of the transgenic *Populus* trees was also shown to be reversed by gibberellic acid application (Han et al. 1997). Unlike the COMT-suppressed *Populus* wood (Van Doorsselaere et al. 1995, Tsai et al. 1998, Lapierre et al. 1999), the COMT suppression found in the present study was not accompanied by red wood coloration in 35S-PtCOMT *B. pendula* lines. This may indicate either differences among the species or the low or moderate level of COMT enzyme suppression.

Histochemical GUS assay and microscopical analysis of transgenic plants with PtCOMTp- or PtCOMTdp-promoters (II)

The two PtCOMT promoters, PtCOMTp- and PtCOMTdp, had the strongest seasonal activity in the new xylem of *B. pendula* stem wood between the 8th and 13th weeks from the beginning of the growing season. The promoter activities started to decline from the 18th week on. Correspondingly, in xylem samples of *Populus* stem wood the COMT enzyme activities peaked approximately on the 8th and 12th weeks from the beginning of the growing season, declined sharply after the 13th or 14th week, and had mostly disappeared by the 18th week (Bugos et al. 1991, Meng & Campbell 1998). In the *B. pendula* roots, on the other hand, the period of strong PtCOMT promoter activity occurred later (on the 13th week) and for a shorter period of time, thus indicating a slightly different seasonal pattern compared to that of the stem wood.

The strong PtCOMT promoter activities in developing xylem support the active role of COMT in ongoing lignification, as demonstrated in previous studies in *Eucalyptus gunnii* (Hawkins et al. 2003) and *P. tremuloides* (Bugos et al. 1991), (Prof. W. Boerjan, Univ. of Ghent, Belgium, pers. com). In *B. pendula,* the PtCOMT promoters induced expression in all xylem cell types without any pronounced differences between fibres, vessels and rays. This is in contrast to the preferential *COMT* gene expression in *P. tremuloides* xylem fibres (Prof. W. Boerjan, Univ. of Ghent, Belgium, pers. com), suggesting a need for COMT-regulated

methylation for S unit synthesis in fibre cell walls known to have a high S unit content (Fergus & Goring 1970, Saka & Goring 1988). In *E. gunnii*, the cell-specific COMT expression pattern varied according to the developmental status of the stems, whereas in immature stem internodes the COMT expression was associated with vessels, and in internodes of a more mature state the COMT expression was present in all xylem cell types (Hawkins et al. 2003). The results suggest potential differences in COMT expression both between the woody angiosperm species and developmental state of the plant.

In stem hair cells the transient GUS expression regulated by the PtCOMTdp-promoter is most probably related to the biosynthesis of phenylalanine-derived compounds other than lignin. In *P. tremula* x *P. alba* stems, the CCoAOMT promoter driven GUS expression seen in hair cells was assumed to be due to the biosynthesis of lignans (Chen et al. 2000), which are derived from the same monolignol precursors as lignin and are associated with the protective functions in plant cells (MacRae & Towers 1984, Harmatha & Nawrot 2002)

The GUS expression driven by PtCOMT promoters was strongly induced in the phloem and xylem of tension wood formed as a result of mechanical stem bending. In tension wood, the content of S units of lignin is higher than in normal wood lignin (Sarkanen & Hergert 1971), and S units may be present in the G layer characteristic to tension wood (Joseleau et al. 2004). When the transcripts of mechanically stressed *Eucalyptus* wood were profiled, *COMT* exhibited a more distinct expression pattern than that of several other lignin biosynthetic genes that may be related to the changes in cell wall composition during bending stress (Paux et al. 2005). Corresponding to the PtCOMT promoter activities in stems of *B. pendula*, the expression of *COMT* (Prof. W. Boerjan, Univ. of Ghent, Belgium, pers. com) and CCoAOMT promoter activities (Chen et al. 2000) were also induced by tension wood formation in *P. tremula* x *P. alba*. On the other hand, it seems that PtCOMT promoter activities and thus S lignin biosynthesis are not necessary to compensate for the wounding in *B. pendula* stem wood. In wounded *E. gunnii* wood, the content of S units was lower, and its S/G unit composition therefore also differed from developmental lignin (Hawkins & Boudet 2003).

The lack of major differences between the PtCOMTp- and PtCOMTdp-promoters suggest that the deleted promoter sequence (1-471 bp) did not contribute significantly to the regulation of gene expression in the present material.

The effects of the PtCOMT modification on insect herbivores (III)

In the leaf-feeding experiments on leaves of PtCOMT-modified birches, larvae of *T. carpinata* and adults of *A. alni* and *Phyllobius* spp. selected transgenic and control leaves equally. The larvae of *A. punctulata* and *C. cinctaria* consumed more leaves of line 130 and *A. punctulata* also more leaves of line 23 compared to the control. The preference for leaves of line 130 may be due to the delayed growth rhythm of line 130, resulting in the younger and thus more preferential leaf quality for feeding. The preference for leaves of line 23 by *A. punctulata* larvae is presumably not associated with a decreased S/G of leaf lignin since there was no preference for leaves of the other 35S-PtCOMT line (44) with comparable lignin modifications. Leaf suitability for larval growth may, for instance, be related to the high content of water and nutritious compounds (sugars and proteins) and/or to the low content of defensive compounds such as foliar phenols e.g. in *Betula* species (Haukioja 2005, Treutter 2006).

The relative growth rates of the lepidopteran larvae did not differ significantly between the control and transgenic birch lines. The lowest growth rates of *A. punctulata* and *T. carpinata*, on the other hand, were measured on leaves with a decreased S/G ratio (lines 23 and 44), which could be due to the limited cell wall degradation and thus carbohydrate and/or protein

utilisation associated with the increased G lignin content (Jung & Deetz 1993, Baucher et al. 1998, Lam et al. 2003). Since a low lignin content is associated with the improved forage digestibility (Sewalt et al. 1997, Guo et al. 2001, Barriére et al. 2003), the best RGR of *A. punctulata* and *C. cinctaria* larvae on leaves of line 130 may be due to the decreased leaf lignin content. Furthermore, the ability of lepidopteran larvae to consume leaf veins, in addition to mesophyll, was not affected by the PtCOMT modification. In general, because the majority of leaf lignin is deposited in veins (Stafford 1988), it can be assumed that species such as lepidopterans are more affected by the modified lignin supplied in their diet in contrast to species with a pronounced ability to feed on mesophyll.

The effects of the PtCOMT modification on the ectomycorrhizal interaction between *B. pendula* and *P. involutus in vitro* (IV)

P. involutus was able to form ECM with *B. pendula* control clones and all the PtCOMT-transgenic lines. Similarly, the 4CL modification did not affect the ability of *B. pendula* to form ECM with *P. involutus in vitro* (Seppänen et al. 2007). In our study, the number of lateral roots covered with fungal hyphae and/or characteristics of an HN showed transgenic line dependent variation compared to the respective control clone. Inoculation with ECM fungus is known to enhance the early growth of woody plants *in vitro* (Grellier et al. 1984, Niemi et al. 2004), and the positive impact of *P. involutus* on the viability and root and shoot growth of the majority of the *B. pendula* clones and lines *in vitro* support this view. The improved plant growth may be a consequence of the increased formation of lateral roots in the presence of the fungus (Béguiristain & Lapeyrie 1997, Tranvan et al. 2000, Niemi et al. 2002a, Niemi et al. 2002b), thus increasing the efficiency of nutrient and water uptake by the plant.

In general, the individual ECM characteristics could be directly or indirectly related either to the lignin modification or to the position, epistatic, or pleiotrophic effects of transgenes. In lines 110 and 130 the root growth was retarded due to genetic transformation, and the subsequent formation of an HN was poor. The poor formation or absence of an HN may contribute to the less efficient nutrient exchange between the symbionts, thus resulting in unchanged plant growth despite the inoculation, as observed in lines 110 and 130. On the other hand, the plant may benefit from the fungus in the absence of an ECM (Niemi et al. 2000, Niemi & Häggman 2002), e.g. due to specific plant growth regulators released by the fungus or advantageous modification of the medium composition (Niemi et al. 2004, Niemi et al. 2007), which could explain the strongly increased proportion of viable plants among lines 110 and 130.

Line 23, which had a decreased S/G ratio of root lignin, formed less lateral roots covered with fungal hyphae and had a less developed HN than the control clone when inoculated with *P. involutus*. Lignification in the ECM association could contribute to reinforcement of the cell walls and formation of a physical and chemical barrier limiting the presence and penetration of the fungus in the intercellular space in the same way as for attack by fungal pathogens (Bucciarelli et al. 1999, He et al. 2002, He & Wolyn 2005). Recently, fungal infection in wheat has been shown to induce a defence-response in the cell walls and involves the enrichment of syringyl lignin solely and a corresponding shift in S/G (Menden et al. 2007). The presence of genes for ligninolytic activities have also been identified for a range of ECM fungi including *P. involutus* (Chen et al. 2001), but whether the lignin-degrading enzymes contribute to ECM associations is still not known. In the *P. involutus-B. pendula* ECM, induced expression of the lignin-related genes *CCoAOMT, SAD* and a gene homolog to dirigent protein was assumed to be associated with the host's defence response to the fungal inoculation (Le Quéré et al.

2005). The slightly different S/G ratios between the non-inoculated and inoculated roots in our study suggest a potential interaction between ECM formation and monolignol synthesis, although no unambiguous effect was observed.

CONCLUSIONS

The introduction of 35S-*PtCOMT* and UbB1-*PtCOMT* transgenes into the *B. pendula* resulted in stable integration into the plant genomes, and a variable expression of transgenes, and a modified lignin composition was observed in the 35S-PtCOMT lines. The results demonstrate the flexibility of lignin biosynthesis and support the essential role of COMT in S unit pathway. The unchanged morphology and growth characteristics of the 35S-PtCOMT-modified *B. pendula* lines support the ability of plants to tolerate a large variation in the lignin S/G ratio. Our understanding of the natural variation of lignin composition in e.g. *Betula* species is deficient, and we therefore cannot exclude the possibility that the modified lignin in the 35S-PtCOMT lines falls within the natural variation.

The PtCOMT-promoter driven GUS expression in the new xylem and lignified phloem fibres during the active growth period of *B. pendula* corresponds well to the essential role of COMT in lignification. The similar reduction in S lignin synthesis in the leaves, stem wood and roots indicates that lignin biosynthesis is under uniform regulation in these organs. The PtCOMT-promoter analyses further suggest that COMT plays a role in lignin formation during tension wood formation, but not in response to wounding. The involvement of transcription factors such as MYB and/or lignification suppressors in these differential responses remains to be elucidated.

Currently, we are only starting to understand the complex ecological impacts of lignin-modified trees. The potential ecological effects of the PtCOMT-modified *B. pendula* plants were investigated with respect to the feeding performances and relative growth rates of common leaf-feeding insect herbivores, as well as the ectomycorrhizal interaction with *Paxillus involutus in vitro*. The results indicated that clone or line does have an effect in these interactions, and that the different characteristics observed in specific PtCOMT lines were either associated with lignin modification or potentially resulted from the position, epistatic or pleiotrophic effects of transgenes. The species used in the present *in vitro* and greenhouse experiments represent a small but specific section of all the potential interactions existing *in vivo*. Therefore, more experimental field assays with a variety of species including mammalian herbivores and decomposer organisms are needed to obtain a more comprehensive picture.

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