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Field trials with lignin-modified transgenic trees

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Abstract

For the last 20 years, as a complement to biochemical approaches, lignin-modified transgenic trees have proven to be invaluable tools for deciphering the lignin biosynthetic pathway, and increasing our knowledge about the role of lignins in wood specific properties important for tree physiology. In addition, their evaluation in the field has been very useful for the identification of possible routes to improve wood properties for specific industrial uses, initially pulp and paper-making and now biofuel production. In this paper, we review our present knowledge gained through numerous studies analysing transgenic trees, with emphasis on field trial evaluations, for assessing the effects of lignin modification on wood properties and tree physiology. All the data presented here led to the conclusion that, prior to use in forestry for pulp and paper or bioenergy production, the fitness of lignin-modified transgenic trees required careful evaluation in a variety of field environments. Although limited in number, environmental studies failed to indicate any strong effects of lignin-modified transgenic trees on the ecosystem. Finally, this review helps to identify some promising options where biotech trees could be used to further optimize the production of wood for human uses.

Keywords: Transgenic tree; Field trial; Lignin; Growth and development; Environmental impact; Pulp and paper; Biofuel

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I. Introduction: lignin a major wood polymer

Wood is a renewable resource that has always been used by mankind providing energy, timber and fibers. Beside these traditional uses, wood appears nowadays as an attractive source of material for biofuel production.

For the tree, wood is a complex tissue that fulfills different vital functions important for its growth and development in a changing environment. Indeed, xylem vessels and tracheids conduct xylem sap, transporting water and mineral elements from the roots to the crown, making possible the large spatial development of trees. In addition, xylem fibers confer to the trunk and branches the mechanical strength necessary to support over the year the ever-increasing weight of the tree and to withstand environmental factors such as wind and snow that may compromise tree equilibrium. To be able to fulfill these functions, wood exhibits very complex features regarding its structure and chemical composition, that may be adjusted according to tree development and variations in environmental conditions. Wood cell walls are organized as a multilayer structure resulting from the assembly of different constituents. This organization may be assimilated to a composite material made of rods of crystalline cellulose microfibrils glued into a matrix of lignins, hemicelluloses and pectins.

Lignin is a major component of wood, ranging from 15 to 36% of its dry weight (Zobel and van Buijtenen, 1989): it is, after cellulose, the second most abundant natural polymer on earth. Lignin is deposited in the cell wall at the end of xylem cell differentiation. Lignin is important for two main functions of wood in trees, *i.e.* xylem sap conduction and mechanical support. Indeed, lignin deposition reinforces plant cell walls, providing rigidity, impermeability to water and also protection against pathogens. Therefore, lignin biosynthesis appears in vascular plants as a critical innovation for plant adaptation to an aerial environment and further to the development of trees (Kentick and Crane, 1997). Lignin is a highly variable molecule, whose synthesis appears influenced by environmental cues as well as by physiological parameters such as tree age, cell type or cell wall layer (Campbell and Sederoff, 1996). Therefore, lignin synthesized by a young tree grown for several months in the sub-optimal conditions of a greenhouse can be rather different from the lignin synthesized by the same tree after several years of growth in the field.

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Lignin is a complex and variable aromatic polymer resulting from the polymerization of elementary units, namely *p*-hydroxycinnamyl alcohol monomers or monolignols, differing by the number of methoxylations on their aromatic cycle: this is why subsequently, in accordance to Sarkanen and Ludwig (1971), we prefer to use the term “lignins” indicative of an array of different polymers, in place of the term “lignin” indicative of a single lignin molecule. In gymnosperms, lignins result mostly from the incorporation of coniferyl alcohol (producing guaiacyl (G) units) and a small proportion of *p*-coumaryl alcohol producing *p*-hydroxyphenyl (H) units. In angiosperms, lignins are composed approximately equally of G and syringyl (S) units resulting from the incorporation of sinapyl alcohol. The proportion between the different elementary units affects their linkage within the polymer and in consequence the structure and properties of the lignin polymer. In accordance, lignins will be more or less condensed, and in consequence, more or less resistant to chemical attacks, natural (pathogen) or artificial (delignification during pulp production).

Lignin biosynthesis has been extensively studied over the last 50 years (Sarkanen and Ludwig, 1971 and for recent reviews see Boerjan *et al.*, 2003, Bonawitz and Chapple, 2010). In poplar, most if not all genes involved in the biosynthesis of lignins have been identified, even before the publication of the poplar genome sequence (Tuskan *et al.*, 2006). The availability of the poplar genome sequence enabled the complete identification of all members of each lignin gene family, even though it still remains to be elucidated for many the specific function of each of these members, whether they are specifically expressed in a given tissue, a cell-type, a stage of development or in response to a stress, or if they encode enzymes with different substrate specificities...

Briefly, monolignol synthesis derives from the phenylpropanoid pathway, which is initiated by deamination of phenylalanine by phenylalanine ammonia-lyase (PAL). The lignin building blocks result from a series of hydroxylation and *O*-methylation reactions on the aromatic ring and the conversion of the side-chain carboxyl to an alcohol group (Boerjan *et al.*, 2003). For a long time, it was thought that the modifications on the aromatic ring occurred at the level of free hydroxycinnamic acids, but recent discoveries led to a reformulation of the pathway (see for details, Bonawitz and Chapple, 2010). The monolignols are then transported through the plasma membrane to the extracellular matrix where it is believed that peroxidases and/or

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laccases initiate their polymerization. At the present time, little is known about the modalities of monolignol transport and polymerization (Li and Chapple, 2010). In addition, we do not yet really know whether this synthesis is strictly cell autonomous or if lignin precursors can be translocated from living adjacent cells, such as xylem ray parenchyma cells (Feuillet *et al.*, 2005).

For the last 20 years, the study of transgenic plants modified for genes coding for enzymes of the lignin biosynthetic pathway has efficiently complemented our previous knowledge acquired through biochemical experiments, providing invaluable information on the lignin biosynthetic pathway and illustrating the high plasticity of the lignin polymer. These studies were performed on tobacco and Arabidopsis, but also on trees, merely on poplar but also, more recently, on a few other species such as eucalyptus and pine. We will focus here on the results obtained from transgenic trees with modified lignins with an emphasis on studies involving field trials. Field trials present several advantages when compared to greenhouse experiments (Pilate *et al.*, 1997). In a field trial, tree development is not limited by physical constraints such as pot size for the root, or greenhouse height for the stem. Running field trials allows tree growth and development to be evaluated in more natural conditions and also makes possible, after several years, the production of wood in sufficient quantity for technological assessment of potential modifications to properties. Stability of modifications can be monitored on long-term field trials by regular measurements of easily scorable effects of the genetic modification (for example, a colored phenotype). It is also possible to study the potential effects of the genetic modification on some elements of the ecosystem, such as trophic interactions with herbivorous insects or fungal pathogens, or the effect of wood decomposition rate on carbon flux.

As a matter of a fact, published field studies with transgenic trees are far from numerous for several reasons. The first and probably main reason is certainly related to the time and effort needed to set up a field trial with trees and the corresponding delays in the production of valuable results, delays often not compatible with the duration of research funding. The second reason is linked to the additional regulatory burden of disseminating GM plants in the field. This is why most of the published studies concern analyses of greenhouse-grown GM trees. Finally, it should be pointed out that although numerous field trials are currently under

evaluation, available data from these experiments is limited. In the following section, we will present an outline of the experiments involving transgenic trees, emphasizing, when possible, the comparison of the results obtained in the greenhouse *versus* in the field. For more clarity, the results will be classified according to the gene target of the genetic modification.

II. Evaluation of lignin-modified transgenic trees grown in the greenhouse vs in the field

A. PAL (Phenylalanine ammonia-lyase)

PAL is the first enzyme of the phenylpropanoid pathway and catalyzes the deamination of phenylalanine to produce trans-cinnamic acid. Surprisingly, there is no report on transgenic trees modified for PAL expression: probably, this is related to the pleiotropic effects that may be expected from altering the expression of this enzyme, central to plant secondary metabolism. Only Gray-Mitsumune *et al.* (1999) described the pattern of expression of a reporter gene directed by two poplar *PAL* promoters in 2-month old growth chamber-grown transgenic poplar plants. This expression appears to be developmentally regulated with the highest levels of expression in roots, young leaves and stems. In contrast, this level was low in woody stems, and weak or absent in developing secondary xylem suggesting that the corresponding *PAL* genes are not involved in lignin biosynthesis in wood.

B. C4H (Cinnamate 4-hydroxylase)

C4H belongs to the CYP73A group of the cytochrome P450-dependent monooxygenase protein family and catalyzes the hydroxylation of cinnamic acid to *p*-coumaric acid. The *C4H* gene family has been characterized in poplar by localization of the expression of its different members and by the enzymatic specificity of the corresponding proteins (Lu *et al.*, 2006). As described subsequently, a *C4H* promoter was further used in transformation experiments to target the expression of other transgenes to the xylem cells during secondary cell wall differentiation.

Bjurhager *et al.* (2010) produced and analyzed transgenic poplars down-regulated for C4H expression. The analysis was performed on 1.5m high greenhouse-grown trees. Transgenic trees presented a 30% reduction in Klason lignin content with no significant change in

syringyl to guaiacyl (S/G) ratio. The transgenic plants exhibited normal wood anatomy and were not different from wild-type plants, except for a slight reduction in growth height. The lignin reduction resulted in a 10% decrease in wood density that may be related to a looser arrangement in microfibril cellulose aggregates observed by atomic force microscopy. Analyses of mechanical properties revealed a slight decrease in axial stiffness in the wood of transgenic plants.

The *C4H* gene has also been incorporated in sense and antisense orientations in an elite tree of a commercially valuable eucalyptus species, *Eucalyptus calmadulensis* (Chen *et al.*, 2001), and 100 plants transferred to the field; the authors have not yet reported on the evaluation of the field-grown transgenic trees. Recently, ArborGen also announced the field evaluation of 2 year-old eucalyptus with *C4H* reduced activity. Field-grown transgenic trees with a 20% lignin reduction presented normal or near normal growth. The polysaccharide content was not modified in transgenic trees, but lignin reduction in transgenic trees correlated, in technological tests, with a better release of sugars, resulting in an increased pulp yield with less chemicals and a greater accessibility of cellulose for biofuel production (Hinchee, 2011).

C. 4CL (4-Hydroxycinnamoyl CoA ligase)

4CL is responsible for the CoA esterification of *p*-coumaric acid in *p*-coumaroyl CoA (Bonawitz and Chapple, 2010). Hu *et al.* (1998) characterized two genes encoding *4CL* in *Populus tremuloides* and, according to their pattern of expression, identified one of them, *Pt4CLI*, as specific for lignin biosynthesis in developing xylem tissues. The promoter of this gene was further used to target expression of the transgene to xylem cells at the lignification stage.

This *Pt4CLI* gene was mobilized in a transformation vector, under the control of a strong constitutive promoter, to produce by antisense inhibition transgenic *P. tremuloides* with down-regulated 4CL expression (Hu *et al.*, 1999). Ten-month old greenhouse-grown transgenic trees exhibited more than 90% inhibition of 4CL activity, leading to a 40 to 45% lignin reduction, with no important modification in its composition. Lignin reduction appeared to be compensated by a 15% increase in cellulose. Therefore, the 4CL down-regulation in the most severely affected lines resulted in a cellulose / lignin ratio of 4, twice as much as in the wild type plants: however, this material was not further analyzed for

technological properties. The authors hypothesized that 4CL was involved in a regulation mechanism, specific to trees, coordinating cellulose and lignin deposition. Interestingly, stem, leaves and roots of transgenic plants had enhanced growth, when compared to wild-type plants. Anatomical studies indicated that it was caused by increased cell proliferation activity rather than increased cell size. Due to the very promising results reported in this first study, a number of other experiments targeting the 4CL gene were undertaken and led to the installation of different field trials to evaluate the effects of 4CL genetic modification in natural conditions. Unfortunately, they failed to reproduce the initial results entirely.

In one of these studies, new 4CL down-regulated transgenic poplar lines were produced using the same *Pt4CL1* coding sequence, but using the *Pt4CL1* promoter, which specifically drives gene expression in the developing xylem, in place of the CaMV 2x35S promoter used in the former study. In these conditions, similar modifications were obtained for lignin and cellulose content (Li *et al.* 2003a; Hancock *et al.*, 2008). However, the transgenic plants did not exhibit any increased growth, suggesting that the growth enhancement observed in the former study resulted from pleiotropic effects linked to the use of the CaMV 2x35S promoter known to confer a strong, constitutive expression.

A similar construct was also transferred into triploid Chinese white poplar (*Populus tomentosa*) with again no visible differences in tree growth and development (Jia *et al.*, 2004). Repression of 4CL expression resulted in a major reduction of lignin content (up to 41%) in transgenic poplars, but with no significant difference in polysaccharide content between transgenic and wild-type poplars leading the authors to question whether 4CL is really a control point between lignin and carbohydrate biosynthesis. After peeling the bark, the developing xylem presented a red-brown phenotype.

Recently, Wagner *et al.* (2009) produced and analyzed 2-year-old *Pinus radiata* plants with strong reduction in 4CL activity: transgenic plants exhibited large decreases in lignin content ranging from 36% to 50% reduction, associated with a decrease in G/H ratio, and also some alterations in carbohydrate metabolism, notably a 2-fold increase in galactose content. Both the decreased G/H ratio and increased galactose content were probably related to an increased formation of compression wood. These important modifications at the molecular level translated at the cellular/tissue level in more or less collapsed tracheids, and the appearance of

bands of axial parenchyma inserted within xylem tracheids. At the plant level, the genetic modification resulted in severely dwarfed plants.

In another study, the *Pt4CL1* promoter-driven antisense copy of *Pt4CL1* gene was introduced into the hybrid *Populus tremula* x *Populus alba* INRA 717-1B4 clone and 14 different transgenic lines with reduced *4CL* expression were planted in the field (Voelker *et al.*, 2010). Analyses were performed on trees after 2 years of growth. Most transgenic trees appeared affected in their lignin metabolism with lignin reductions ranging from less than 10% to up to 50% with a reduced S/G ratio and a small increase in H units incorporated into lignins. Transgenic trees appeared diversely affected in their growth and development: it seemed that, after reaching a threshold in lignin reduction, the trees developed strong phenotype alterations. The most affected trees exhibited a red-brown wood phenotype, which was stronger at the basis of the tree, associated with collapsed xylem cells, an important reduction in aerial biomass, modified tree architecture and reduced height. Further in-depth analyses carried out on the same plant material revealed that reduced lignification strongly affected hydraulic and mechanical wood properties. Xylem vulnerability to embolism appeared strongly increased and resulted, in the most affected field-grown trees, to nearly 90% shoot dieback and 25% tree mortality, whereas these events were not observed in the wild type trees or in the same transgenic trees, when grown in the greenhouse (Voelker *et al.*, 2011a). Further analysis of hydraulic architecture on these trees showed that the dependence of xylem resistance to embolism was bound to adequate wood mechanical properties, provided by normal (or near-normal lignin content). In the patches of colored wood which probably had very low lignin content, water transport in vessels appeared strongly impeded, due to the collapse and more importantly the occlusion of these vessels by phenolic deposits and tyloses (Kitin *et al.*, 2010). With regard to mechanical properties, the strong reduction in lignins also resulted in a major decrease in wood strength and stiffness, even though wood density remained comparable to that of control wood. Finally, low-lignin trees were shown to contain up to three-fold more tension wood (Voelker *et al.*, 2011b).

Wood collected from these field-grown *4CL* down-regulated trees were evaluated for bioethanol production: surprisingly, despite their strong lignin reduction and higher cellulose content, pretreated wood did not exhibit any improvement in saccharification efficiency (Voelker *et al.*, 2010). This set of experiments with *4CL* down-regulated trees clearly demonstrated that promising results primarily generated by the evaluation of greenhouse-

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grown *4CL* down-regulated trees (Hu *et al.*, 1999) were not confirmed on trees grown under natural field conditions, i.e. submitted to regular environmental stress. Decreased tree fitness and increased mortality appeared to be linked to a threshold in lignin content making the efficiency of this strategy questionable (strong reduced lignin content) for making wood more amenable to new industrial uses such as biofuel production.

Stout (2011) evaluated for two growing seasons the productivity of a number of transgenic low lignin *Populus trichocarpa* trees (Nisqually-1) with low *4CL* activity planted as short rotation coppice at three different sites in North Carolina, across a range of environmental conditions. In this experiment, *4CL* down-regulated trees exhibited both reduced lignin contents (up to 50% as observed elsewhere) and increased S/G ratios. The tree biomass productivity among trials was extremely variable with more than 10 fold variations between extreme sites; a high field site x transgenic line interaction was evidenced, confirming the need to evaluate transgenic trees at multiple field sites, in the same way as for novel cultivars derived from conventional breeding programs.

Similar to Voelker's study of hybrid aspen (2010), low-lignin trees exhibited decreased xylem specific hydraulic conductivity and increased susceptibility to embolism, due to collapsed cells caused by reduced strength of xylem cell walls, these aspects being exacerbated at the drier sites. However, in this case, *4CL* down-regulation translated in reduced wood density. In addition, wood of transgenic trees with reduced lignin content showed a severe reduction in their modulus of elasticity and their compression strength in the direction parallel to the grain (Hovarth *et al.*, 2010). As observed previously, most low-lignin transgenic trees exhibited loss of apical dominance, stunted growth, stem dieback, and increased sensitivity to pathogens even though both gas exchange and photosynthesis rate were not compromised in low-lignin trees. However, trees from one transgenic line with a strong (37%) lignin reduction produced more biomass than the control at the Coastal Plain site, and as much biomass as the control at the other two less favorable sites. This may be linked to the high number of sylleptic branches produced by the trees of this transgenic line as this number was shown to be indicative of highly productive clones (Marron *et al.*, 2006). In conclusion, this field study with *4CL* down-regulated trees suggests that, even though most low-lignin transgenic trees appeared strongly affected in their fitness even in favorable site, it appears possible to find some transgenic events where the modification may still be interesting for technological applications without apparently affecting the growth and development of the tree. Such identification requires the

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evaluation of a large array of different transgenic events exhibiting large variations in the effects of the genetic modification (as previously suggested in Busov *et al.*, 2005).

To summarize the results obtained on *4CL* down-regulated transgenic poplars, it appears that the use of the same construct in different poplar species led to strikingly different results e.g. *pPt4CL1-ASPt4CL1* led to strongly affected growth and development in *P tremula x P alba* whereas it has no such effects in a related species such as *P. trichocarpa*. In this case, it seems preferable to use a xylem-specific promoter rather than a constitutive pCaMV 35S, even though the *pPt4CL1* promoter does not seem to be strictly xylem-specific (Déjardin *et al.*, unpublished results).

As for *C4H*, there is currently a project in Taiwan aiming to down-regulate *4CL* in *E. camaldulensis* by genetic transformation of an antisense eucalyptus *4CL* gene driven by a xylem-specific promoter. Five different transgenic lines have been produced with a 5~18% lignin reduction as compared to the wild-type plants. In addition, transgenic plants have been planted in the field in order to monitor tree growth performance, to evaluate variations in wood composition and to analyze pulping property of wood chips
http://grbsearch.stpi.narl.org.tw/GRB/result.jsp?id=1358296&plan_no=97%E8%BE%B2%E7%A7%91-1.1.1-%E6%A3%AE-G1&plan_year=97&target=plan&highStr=&check

D. HCT (Hydroxycinnamoyl-CoA:shikimate/quinat hydroxycinnamoyltransferase) and C3H (*p*-Coumarate 3-hydroxylase)

The first HCT was recently purified and the corresponding gene cloned from tobacco (Hoffmann *et al.*, 2003) and later on in *P. radiata* (Wagner *et al.*, 2007). This enzyme converts *p*-coumaroyl-CoA and caffeoyl-CoA to their corresponding shikimate or quinate esters and catalyzes the reverse reaction as well. These shikimate and quinate esters of *p*-coumaroyl-CoA have been shown to be preferred substrates for C3H, which belongs to the CYP98A group of the cytochrome P450-dependent monooxygenase family. This enzyme converts these substrates into their corresponding caffeoyl esters (Schoch *et al.*, 2001) leading to the synthesis of G and S monolignols. Due to the relatively recent isolation of these 2 enzymes, only a few publications report on the evaluation of transgenic trees affected in the expression of either of these genes and none of these studies deals with field trials.

RNAi-mediated silencing of a *P. radiata* *HCT* gene resulted in a strong decrease (up to 42%) in lignin content, a strong increase in H unit (from traces up to 31%) monolignol composition and in modifications in the lignin polymer structure (Wagner *et al.*, 2007): these observations were carried out on pine tracheary elements as transgenic plants were not regenerated.

In the most affected transgenic lines, RNAi suppression of C3H in hybrid poplar (*P. alba* x *Populus grandidentata*) led to reduced (up to 55%) lignin content (Coleman *et al.*, 2008a) and altered S/G/H lignin monomer ratios, resulting from a decrease in G monomers compensated by an increase in H units, while, surprisingly, S lignin remained relatively constant. Likewise, lignin modifications were associated with concomitant increases in cellulose and arabinoxylan (a common hemicellulose from angiosperm cell walls), as well as an increase in the pool of different phenyl glycosides. While irregular patterning of lignin deposition occurs all along the stem, lignification was most notably reduced around vessel elements. Overall, these modifications resulted in plants with impaired plant growth both in height and diameter, with smaller stems, reduced root biomass and altered leaf morphology and architecture (Coleman *et al.*, 2008b). These altered phenotypes at least partly resulted from a strongly reduced cell wall lignification and its consequences, as already observed in *4CL* down-regulated trees: collapsed xylem vessels, reduced hydraulic conductivity and higher susceptibility to embolism. Despite these effects, the authors demonstrated that water transport was not the factor responsible for the observed decreased growth in these transgenic plants. The main reason postulated by the authors was due to the limitation of photosynthesis as a consequence of the reduction in carbon sink strength: this resulted in an accumulation of photosynthates as starch and soluble sugars in the leaves, leading to a decreased rate of photosynthesis and a reduction in stomatal conductance (Coleman *et al.*, 2008b). These observations were carried out on 4-18 month-old greenhouse-grown trees, whereas dramatic growth reductions were observed only 6 weeks after acclimatizing. The defects in water transport observed in these young plants would undoubtedly become even more serious in bigger trees making the evaluation of such material useless in field conditions.

E. F5H (Ferulate 5-hydroxylase)

F5H, also designated coniferaldehyde 5-hydroxylase (CAld5H) is a cytochrome P450-dependent monooxygenase of the CYP84A group. In *in vitro* experiments, it preferentially

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converts coniferaldehyde and/or coniferyl alcohol to sinapaldehyde and/or sinapyl alcohol, respectively (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999).

Young transgenic poplar trees (INRA 717-1B4 *P. tremula* x *P. alba*) transformed with the *F5H* sense sequence under the control of the *C4H* promoter (expressed in lignifying tissues) exhibited a strong increase in S unit content, up to 85% compared to an average 55% observed in the wild type poplar plants, while lignin content was unaffected (Franke *et al.*, 2000). In tobacco, similar results were obtained using the same construct whereas, when the *C4H* promoter was replaced by the CaMV 35S “constitutive” promoter, no changes in S/G ratio were observed suggesting that the 35S promoter does not confer high levels of expression in lignifying tissues. Analyses performed on one year-old transgenic poplar plants from the same transgenic lines found even stronger increases in S units, up to 93.4% compared to 65.6% for control trees (corresponding to an increase S/G ratio from 1.9 in control to 14.2 in the transgenic plants, Huntley *et al.*, 2003). This clearly demonstrated that *F5H* is a key-enzyme controlling lignin monomer composition in angiosperms. Two year-old greenhouse-grown transgenic poplar plants exhibited no change in total lignin content, no apparent effect on conduction and no alteration in their growth and development (Huntley *et al.*, 2003). However, mechanical evaluation of transgenic wood revealed significant increases in elastic modulus, whereas bending stiffness remained unchanged, due to a reduction in stem diameter (Koehler and Telewski, 2006). Likewise, preliminary analyses found higher wood density compared to control wood material. Furthermore, technological analyses revealed that paper of equal or better quality could be made from pulp fiber derived from p*C4H-F5H* transgenic trees, due to a higher degree of polymerization of the residual pulp cellulose, while yielding significant savings in pulping process time and chemicals both for pulping and bleaching.

In a recent communication at the International Scientific Conference on Hardwood Processing (October 16-18, 2011 Virginia Tech, Blacksburg, VA, USA), Maud Hinchee reported ArborGen analyses for the properties of wood collected from 4 year-old transgenic over-expressing *F5H* eucalyptus trees grown in the field. These analyses indicated a S/G ratio of 4.6 in the transgenic wood compared to 2.3 in the wild type. This increased ratio appeared positively correlated to soluble lignin content and to the production of good quality pulp using a smaller amount of alkali (decrease from 21% used for wild type wood down to 16.5%) that

resulted in a 15% reduction in chemical cost and also an improved pulp yield (Hinchee, 2011).

F. CCoAOMT (Caffeoyl CoA *O*-methyltransferase)

CCoAOMT catalyzes methylation of caffeoyl CoA to generate feruloyl CoA (Pakush *et al.*, 1989). Two *CCoAOMT* genes were identified in poplar, and characterized for their expression pattern detected either by immunolocalisation or by promoter activity in poplars transformed by *CCoAOMT* promoter-*uidA* fusion (Chen *et al.*, 2000): both genes were expressed similarly in xylem, but differentially in phloem; they were also both induced after wounding, fungal infection, and bending. Further, the same research team produced transgenic poplars with strongly reduced CCoAOMT activity resulting, as analyzed in 6 month-old greenhouse-grown plants, in a small decrease in Klason lignin content (up to 12%), a slight increase in S/G ratio and a strong accumulation of soluble phenolic acid glycosides (Meyermans *et al.*, 2000). These modifications were associated with a pink-red coloration of the active xylem and an enhanced fluorescence of mature vessel cell walls, with no apparent effects on tree growth and development. Two transgenic lines with strong and stable *CCoAOMT* down-regulation are presently evaluated in the field in France: these trees exhibit normal growth and development.

Zhong *et al.* (2000) also generated transgenic poplar plants with reduced CCoAOMT activity using an antisense strategy. Interestingly, both studies were performed on the same poplar clone (INRA 717-1B4 *P. tremula* × *P. alba*) with *CCoAOMT* poplar sequence (in sense or antisense orientation), and a strong constitutive promoter. Six to nine month-old greenhouse-grown plants exhibited a 40% reduction in Klason lignin content, associated with both a 70% reduction in CCoAOMT activity and an orange coloration of the active xylem. Further analyses using FTIR indicated that the reduction in lignin content led to less condensed and less cross-linked lignin structure in wood. Lignin modifications in the transgenic poplar plants did not result in any abnormal growth pattern even though anatomical observations revealed the occurrence of cell wall distortions for a small proportion of the vessels.

In a recent paper, Wei *et al.* (2008) reported the evaluation of 3-year-old field-grown transgenic poplar (*P. tremula* × *P. alba*) with depressed CCoAOMT activity. Overall, lignin modifications appeared similar to those observed by Meyermans *et al.* (2000). In the Kraft

pulping assay, improved pulp characteristics and an increased pulp yield were observed with the transgenic wood when compared to the wild type wood. The authors did not observe any obvious adverse effects of *CCoAOMT* down-regulation on the growth and development of the lignin-modified trees in field conditions.

In conifers, a 90% reduction of *CCoAOMT* expression in *P. radiata* tracheary element cultures resulted in a 20% reduced lignin content due to depletion of G units, an altered lignin polymer with a 10-fold increase in H/G ratio and the presence of catechyl units due to incorporation of caffeyl alcohol in the lignin polymer (Wagner *et al.*, 2011).

G. COMT (Caffeic acid *O*-methyltransferase)

COMT was previously thought to be a bi-functional enzyme, catalyzing the methylation of both caffeic and 5-hydroxyferulic acids. However, *in vitro* COMT enzymatic assays in the presence of a mix of its different potential substrates later demonstrated that, in *in vivo* conditions, the preferential substrates were 5-hydroxyconiferaldehyde and also possibly 5-hydroxyconiferyl alcohol, giving rise to sinapaldehyde and sinapyl alcohol, respectively (Osakabe *et al.*, 1999; Parvathi *et al.*, 2001).

COMT-down-regulated transgenic poplars (INRA 717-1B4 *P. tremula* x *P. alba*) were produced with a *P. trichocarpa* x *P. deltoides* *COMT* antisense sequence under the control of the pCAMV 35S “constitutive” promoter (van Doorselaere *et al.*, 1995). Three month-old greenhouse-grown plants with a 95% reduction in *COMT* activity showed a very strong S/G ratio reduction (from 1.5 in control plants to 0.25 in the most severely affected line) and an appreciable amount of 5-hydroxyguaiacyl units: the strong increase in the proportion of G in the lignin polymer strongly alters its structure, with an increased frequency of resistant interunit bonds and, conversely, a lower frequency of labile β -O-4 bonds (Lapierre *et al.*, 1999). The lignin content was not affected by the genetic modification, whereas the active xylem consistently showed a pale rose coloration. Interestingly, comparison of lignin characteristics between 4 month-old and 2 year-old plants indicated that lignins deposited at the early stage of lignification had fewer labile β -O-4 bonds in both wild type and transgenic trees, whereas *COMT* down-regulation resulted into a 2-fold increase in resistant 5–5 biphenyl structures (Lapierre *et al.*, 1999). Likewise, the reduction in S/G ratio in 2 year-old transgenic poplars (2-3x) was not as important as in younger plants (6-7x). Overall, the effects

of *COMT* down-regulation on lignin structure proved to have a detrimental impact on the pulping performances of 2-year-old transgenic poplars (Lapierre *et al.*, 1999). This was further confirmed by pulping evaluation performed on wood harvested from 4-year-old trees grown in the field (Pilate *et al.*, 2002). Field-grown transgenic trees were not altered in their growth and development. Interestingly, *COMT* activity and lignin characteristics appeared less affected in these older trees.

Transgenic poplars with extremely reduced *COMT* activity were further produced from the same clone (INRA 717-1B4 *P. tremula* x *P. alba*) by gene silencing resulting from the introduction of a sense poplar *COMT* sequence under the control of the CaMV double 35S promoter, which confers a stronger constitutive expression than the 35S promoter (Jouanin *et al.*, 2000). Six month-old greenhouse-grown trees with a residual *COMT* activity lower than 3% of the control level exhibited a substantial reduction in lignin content (17%) with an almost complete disappearance of S units that makes its lignin polymer comparable to pure G conifer lignins, and also with an important incorporation of 5-hydroxyguaiacyl units (11%). Their wood presented a different brownish coloration while tree growth and development were not affected. Kraft pulping assay realized on this material indicated an increased pulp yield (10%), but a high kappa number indicative of a high residual lignin content in the pulp, showing that the delignification treatment was rather inefficient on this material.

Finally, another study reported the analysis of transgenic aspen (*P. tremuloides* Michx.) with down-regulated xylem *COMT* activity *via* cosuppression by a homologous sense full-length *COMT* transgene (Tsai *et al.*, 1998). The woody stems of transgenic aspen harbored a mottled reddish-brown coloration, somewhat similar to the observation of Jouanin *et al.* (1999). The authors demonstrated that the coloration was due to the incorporation of abnormal amounts of cinnamyl aldehyde residues in lignins. Here, the genetic modification did not affect lignin content, but the S/G ratio appeared strongly decreased, as in the previous studies. The patchy pattern of coloration suggests that the *COMT* gene silencing did not occur evenly along the stem.

H. CCR (Cinnamoyl CoA reductase)

CCR converts hydroxycinnamoyl CoA esters to their corresponding aldehydes, the penultimate step in monolignol biosynthesis.

Down-regulation of CCR was obtained in transgenic poplars (INRA 717-1B4, *P. tremula x P. alba*) by the integration of a sense or antisense copy of a *P. trichocarpa* CCR sequence, shown to be the only one strongly expressed in differentiating xylem (Leplé *et al.*, 2007). About 5% of the transformed plants were dwarf and unable to be acclimatized, suggesting deleterious effects when CCR down-regulation was too strong. Less affected transgenic lines, still with a 3-4% CCR transcript residual level in some wood areas, were able to grow normally in the greenhouse. These plants were shown to be strongly affected in lignin metabolism, with up to a 50% reduction in lignin content. Lignin structure was more condensed apparently due to a decrease in S unit incorporation. In addition, the decreased flux of feruloyl-CoA to lignin resulting from CCR down-regulation appeared to be compensated by an increased flux toward ferulic acid, further detoxified by its glucosylation. Lignin alterations appeared confined in patchy areas, where active xylem exhibited an orange-brown coloration potentially due to the incorporation of ferulic acid in the lignin polymer (Leplé *et al.*, 2007). Accordingly, the cohesion of the cell wall was strongly altered in these areas, with the occurrence of additional concentric sublayers within the S2 layer of fibers. In this respect, it was hypothesized that ferulic acid deposition in the wall was actively induced to strengthen the cell wall. Transcriptomic and metabolomic evaluation found an oxidative stress response likely to be induced by cell wall alterations. These analyses also revealed that, in addition to lignins, hemicellulose and pectin were submitted to increased breakdown or remodeling and decreased synthesis, with a concomitant increase in cellulose content.

CCR down-regulated trees from the most affected lines that were able to grow normally in the greenhouse were evaluated in a field trial. Upon sample collection after 5 years, the colored phenotype was mostly concentrated in the basal part of branches and stems. Wood collected from 4-year-old field-grown trees altered by CCR gene silencing (with a sense construct) appeared easier to delignify than control trees using lower alkali concentrations, although pulp yield was decreased. In addition, the cell wall alterations resulting from the genetic modification were associated with a consistent reduction in tree growth, both in height and diameter. CCR-silenced poplars are still grown in the field, both in France and Belgium (Custer, 2009) for further evaluation of the potential increased ability of their wood to be converted to fermentable sugars in the process for bioethanol production. Preliminary analyses on wood samples from one year-old field-grown coppices provided very encouraging results with ~50% increase in saccharification potential (Vanholme *et al.*, 2011).

Likewise, *CCR*-down-regulated transgenic Norway spruce plants (*Picea abies*) were produced by particle bombardment of embryogenic cultures with a construct harboring the homologous *PaCCR* gene in an antisense orientation under the control of the maize ubiquitin promoter. The most affected five-year-old transgenic plants, with about 60% residual *CCR* transcript levels, exhibited altered lignin contents (up to 8% reduction) and compositions, with a reduced quantity of H units in the non-condensed lignin fraction (Wadenbäck *et al.*, 2008). Five to six-year-old greenhouse-grown transgenic spruce plants appeared normal but had reduced stem diameters (up to 20%) compared to control plants. Furthermore, small-scale Kraft pulp assays carried out with *CCR* down-regulated spruce wood indicated a better delignification with a 3.5% reduction in the kappa number.

I. CAD (Cinnamyl alcohol dehydrogenase)

CAD catalyzes the last step of the monolignol biosynthetic pathway that is the conversion of cinnamyl aldehydes into their corresponding alcohols.

The spatial and developmental regulation of the *CAD* gene was assessed in transgenic poplars (INRA 717-1B4 *P. tremula* × *P. alba*) transformed with a chimeric gene fusion of a eucalyptus *CAD* gene promoter with the *uidA* reporter gene. In greenhouse-grown plants, a specific *GUS* expression was detected in the vascular tissues of stems, roots, petioles and leaves. In addition, *GUS* activity appeared preferentially localized in xylem ray cells and parenchyma cells surrounding sclerenchyma fibers (Feuillet *et al.*, 1995), but also in vessels and fibers undergoing lignification (Hawkins *et al.*, 1997). These transgenic poplar plants were subsequently planted in the field in order to evaluate the pattern of expression of the *CAD* gene in trees grown under natural conditions. This analysis was done on trees derived from 20 different transgenic events. Overall, the tissue specificity of the *CAD* promoter appeared to be conserved and stable in field conditions over an extended period (Hawkins *et al.*, 2003).

Incorporation of a sense or antisense copy of a *CAD* poplar (*P. trichocarpa* × *P. deltoides*) gene in transgenic poplars (INRA clone 717-1B4, *P. tremula* × *P. alba*) resulted in *CAD* down-regulation with 30% residual enzyme activity (Baucher *et al.*, 1996). The developing xylem of

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these plants displayed a strong red coloration, whereas plants with weaker down-regulation exhibited patchy phenotypes, sometimes with a radial pattern. This coloration was shown to result from the presence of conjugated aldehydes in the lignins. The modified plants did not exhibit any visible cell wall alterations. Preliminary analyses on 3 month-old plants did not find important differences in lignin content and composition (S/G ratio) (Baucher *et al.*, 1996). Nevertheless, chemical-pulping tests revealed that the transgenic wood was easier to delignify (up to 22% Kappa number reduction), while the degree of polymerization (DP) for cellulose, pulp yield and fiber length were preserved. These results obtained on very juvenile plants were later confirmed on 2-year-old plants (Lapierre *et al.*, 1999) and finally in 4-year-old field-grown trees planted in two sites, Ardon (France) and Jealott's Hill (United Kingdom) under different climate and soil conditions (Pilate *et al.*, 2002). Unfortunately, the English field trial had an early termination, due to its destruction by environmental activists. Nevertheless, analyses revealed that, in these older plants, the genetic modification resulted in a slight, but consistent, decrease in lignin content (less than 20% compared to wild-type). Further in-depth lignin analyses revealed that *CAD* down-regulation effects on the structure of the lignin polymer were not due to any changes in the frequency of labile ether bonds or guaiacyl units in lignins, but resulted from an increased proportion of syringaldehyde and diarylpropane structures and more importantly in an increased frequency of free phenolic groups (Lapierre *et al.*, 1999). This higher content in free phenolic units facilitated lignin solubilization and fragmentation during Kraft pulping. The most affected antisense line (ASCAD21) presented increased pulping properties: it appeared easier to delignify using less chemicals, leading to a better pulp yield with less cellulose degradation. Regarding growth and development of transgenic trees in the field, no difference in bud burst was observed between transgenic and wild type lines. Likewise, none of the antisense lines exhibited any significant differences in height or trunk diameter. Nevertheless, the only cosuppressed line planted in the field revealed a significant reduction both in primary and secondary growth (Pilate, unpublished results). As this was the only cosuppressed line present in the field trial, we were not able to conclude on a specific effect of cosuppression. At the end of the field assay, 12-year-old poplar wood was collected and will be analyzed in order to determine whether the promising results obtained with the ASCAD21 line will be confirmed on mature wood at the age of commercial harvest. The red coloration in the developing xylem was still present, indicative of the stability of the *CAD* antisense suppression. In addition, the evolution of lignin characteristics will be determined by analyses to be performed within the different annual rings.

In another study, 7-month-old greenhouse-grown transgenic poplars (cv Ogy *P. deltoides* x *Populus nigra*) with very low CAD activity (10% residual CAD activity) were analyzed: the lignins of these plants presented the same characteristics as in the previous studies but were exacerbated (Lapierre *et al.*, 2004). Preliminary small-scale assays indicated that more than 50% of the lignin content was released after very mild alkaline treatment (2N NaOH at 37°C) indicating very promising pulping characteristics for this wood. Unfortunately, these CAD down-regulated plants appeared affected in their growth and development even in favorable greenhouse conditions (Pilate *et al.*, unpublished results).

The results obtained from CAD down-regulated poplars is comparable to the analyses performed on a natural *cad* mutant in loblolly pine. This mutant *cad* allele (*cad-n1*) resulted in severely decreased CAD gene expression and, in homozygous mutant trees, modified wood color and lignin composition (MacKay *et al.*, 1997). *cad-n1* homozygotes had nearly no detectable CAD activity whereas heterozygous plants had half the wild type CAD activity. Free phenolics, mainly coniferaldehyde - the preferred substrate of CAD in conifers - and vanillin showed substantial increases in content (10x) compared to wild type wood. The wood of the *cad-n1* homozygotes, however, was distinctly brown, whereas pine typically has a white wood. Lignin content was not different from wild type in heterozygous plants and only slightly reduced (nearly 10% reduction in the homozygous plants, whereas significant amounts of coniferaldehyde appeared to be incorporated into the lignin polymer of mutant plants leading to a lignin polymer with more condensed inter-unit linkages (such as 5-5 bonds) and lower levels of β -O-4 bonds. Chemical pulp assays indicated that an efficient delignification of wood from the homozygous mutant trees could be obtained with low sulfide levels. Pulping studies with heterozygous wood from juvenile trees found a lower Kappa number, but this did not seem to be maintained in mature trees (Scott *et al.*, 2002).

Under greenhouse conditions, the morphology and growth of *cad-n1* homozygote pine plants could not be distinguished easily from that of other genotypes. However, it was later observed that homozygous *cad* mutant pine trees had extremely low growth rates and a high rate of death (Scott *et al.*, 2002), whereas growth of heterozygous trees was clearly enhanced with a 14% increased volume mostly resulting from increased radial growth (Wu *et al.*, 1999). In fact this genotype was selected sometime ago in Southern US breeding programs for its

increased growth rate. Despite the obvious differences between angiosperm and gymnosperm lignins, it is worthy of noting the numerous similarities in the effects of different levels of *CAD* down-regulation between natural mutants and GM trees.

J. Peroxidases and laccases

Peroxidases and/or laccases catalyze the oxidation of monolignols, the last step in lignin biosynthesis. Using hydrogen peroxide as an oxidant, peroxidases can generate phenoxy radicals from monolignols, which are then coupled to generate lignin polymers. Laccases can promote polymerization of monolignols in the absence of H₂O₂, resulting in either lignans or lignins (Sterjiades *et al.*, 1992).

The expression pattern of a promoter from an anionic peroxidase gene (*PRXA3*) potentially involved in lignin synthesis was determined in transgenic poplars transformed with a construct carrying a fusion of this promoter with the reporter gene *uidA*: the reporter gene appeared strongly expressed in the xylem and weakly in the phloem (Morohoshi and Kajita, 2001). Transgenic poplars (*Populus sieboldii* x *P. grandidentata*) down-regulated for this peroxidase gene were produced using an antisense copy of this gene under the control of its own promoter (Li *et al.*, 2003b). These authors reported that 3-month-old greenhouse-grown poplar trees, with decreased total peroxidase activity in the stem (55% residual activity), exhibited decreased lignin content (up to 20% reduction) and increased S units in the lignin polymer compared to wild type trees. Growth and development were similar in wild type and transformed trees. More surprisingly, poplar (*P. tremula*) transformation with an antisense sequence of a cationic peroxidase gene (*ShPX6a*) from a forage legume species, *Stylosanthes humilis*, under the control of the *35S* promoter, gave a similar 10-20 % reduction in lignin content (Ipekci *et al.*, 1999). None of these transgenic trees was, however evaluated further in a field test.

Poplar plants (INRA 717-1B4, *P. tremula* x *P. alba*) transformed with an antisense copy of three different laccase genes were analyzed for their potential effects on lignification. Despite severe reduction in the expression of each gene in transformants, neither plant growth and development nor their lignin content and composition were altered. Interestingly, repression

of one of these laccase genes (*LAC3*) induced a 2- to 3-fold increase in total soluble phenolic content that was preferentially accumulated in xylem ray parenchyma cells, whereas xylem fiber cell wall structure appeared dramatically altered (Ranocha *et al.*, 2002).

K. Multiple transformations

Based on the results of the single gene transformation approach, several studies were undertaken to try to combine the modification of several lignin genes in a single tree and also to evaluate any possibility of interactions between enzymes from the lignin biosynthetic pathway. This was the case for a combinatorial approach developed by Li *et al.* (2003a), who, using an *Agrobacterium*-mediated cotransformation system, introduced into the same tree both antisense poplar *4CL* and sense sweetgum *F5H* under the control of the xylem specific promoter *pPt4CLI*. Ten-month-old greenhouse-grown double-transformant plants did indeed exhibit both a strong reduction in lignin content reminiscent of *4CL* down-regulation and a strong increase in S unit, characteristics of *F5H* over-expression. Further evaluation of these modified trees under field conditions found, however, alterations in growth and development as observed in *4CL* single transformants. Additive results were also obtained for *ASCAD x ASCOMT* double-transformed poplars (Lapierre *et al.*, 1999). Interestingly, certain other combinations did not yield additive results: this was the case of *COMT x CCoAOMT* double transformants which were unable to develop in field conditions and died during the first winter season after plantation, whereas single transformant trees planted alongside developed similarly to control trees (Pilate, Leplé and Boerjan, unpublished results).

Several attempts have been made to induce the production of S units in a conifer. Toward this end, a large number of larch transgenic lines transformed with a construct carrying a poplar *COMT* gene in the sense orientation under the control of the pCaMV 35S constitutive promoter were produced. However, transgenic larch stems with strong heterologous OMT activity did not exhibit any strong changes in lignin composition (Pilate *et al.*, 1999).

Further, a poplar *F5H* gene under the control of the double pCaMV 35S promoter was introduced into a *COMT* over-expressing transgenic larch line, but the resulting transformed lines did not revealed the appearance of any S unit incorporated in the lignin of transgenic plants (Gatineau *et al.*, 2001). This led to the conclusion that the introduction of these two

enzymes was not sufficient to induce S unit production and incorporation in a conifer background.

Besides genes coding enzymes from the monolignol pathway several other GM trees have been shown to be affected to differing degrees in their lignin content or composition.

L. LIM-domain transcription factors

Eucalyptus (*E. camaldulensis*) plants were transformed with an antisense copy of a LIM domain transcription factor gene from eucalyptus (*EcLIM1*) involved in the regulation of lignin biosynthesis under the control of the CaMV 35S promoter. Suppression of *EcLIM1* expression in 10-month-old greenhouse-grown transgenic plants resulted in a coordinated decrease in the expression levels of several lignin biosynthesis genes, namely those coding for *PAL*, *C4H* and *4CL*, resulting, in the most altered plants, in a 29% reduction of lignin content. The most affected plants presented altered growth with premature leaf loss, possibly due to alterations in water transport ability (Kawaoka *et al.*, 2006).

M. DOF-domain transcription factors

The sequence of a DOF (DNA-binding with one finger) domain transcription factor (named *WALLDOF*) with a cambium-preferred expression profile was introduced into poplar under the control of a *C4H* xylem-specific promoter (Gehhardt *et al.*, 2011). Two month-old transgenic poplar plants were screened for changes in lignin deposition. Overexpression of *WALLDOF* in transgenic poplar led to a dramatic thickening of secondary walls and a reduction in the lumen area. This wall thickening resulted in higher wood density, increased cellulose content, reduction in hemicellulose carbohydrates, mainly mannose and arabinose, and alterations in lignin composition.

N. UDP-glucose pyrophosphorylase

The modification of genes apparently not involved in lignification translated sometimes in strong effects on lignification. This was the case when a bacterial UDP-glucose pyrophosphorylase was over-expressed in transgenic poplar in order to increase cellulose production and content. In effect, in 4-month-old transgenic poplars, the genetic modification induced important pleiotropic effects linked to alterations in C allocation, and resulting in

impaired growth and development, with reduced growth height and stem diameter, elongated axial shoots, and smaller leaves. Further analysis performed on the transgenic poplar plants revealed an increased polysaccharide content, including cellulose, and a concurrent decrease in lignin content, increased S/G ratio and also a dramatic increase in salicylic acid 2-O- β -D glucoside probably associated with plant responses to the stress associated with the genetic modification (Coleman *et al.*, 2007). However, it should be pointed out that the change in lignin content was only the consequence of an increased proportion of cellulose in the cell wall. The explanation for the increased S/G ratio that was linked to an increase in *F5H* transcript abundance is not so straightforward.

O. Synthetic tyrosine rich peptide gene

This pioneer study is based on the hypothesis that the free radical coupling between monolignol subunits and tyrosine will result in a lignin structure that can be easily hydrolyzed with proteases (Liang *et al.*, 2008). Toward this end, a parsley tyrosine-rich hydroxyproline-rich coding sequence glycoprotein gene was fused to a leader sequence derived from a lodgepole pine xylem β -glucosidase cDNA and the poplar *PAL2* promoter to address transgene production to the cell wall during lignification. Greenhouse-grown transgenic poplar (*cv. Ogy P. deltoides* \times *P. nigra*) plants did not show abnormal growth and development, stem structure and lignin content were not affected, whereas transgenic stem stiffness was reduced by 10 to 20% compared to wild type stems. Although, the interaction of tyrosine-rich peptides with phenolic hydroxyl groups was not confirmed, preliminary tests indicated that the transgenic lines were more susceptible to protease degradation and released more sugars than wild type plants (Liang *et al.*, 2008).

III. Evaluation of potential effects of lignin-modified transgenic trees on the environment

Although plants with altered lignin composition may appear robust when grown in a controlled environment, it remains possible that in field conditions, when exposed to biotic (pathogens, herbivores) and abiotic (drought, wind...) stresses, their fitness and even their capacities to develop normally will be affected (Bonawitz and Chapple, 2010). The technological advantages gained through lignin modification may be totally offset by potential reductions in fitness due to negative trophic interactions in regular plantation

environments. Likewise, little is known about any effects of lignin modification on the flux of carbon to the surrounding environment.

A small set of studies have addressed this issue in order to determine if the valuable technological gains from using lignin-modified trees might be offset by environmental issues. This is probably related to the practical difficulties in carrying out such an estimation. Most studies on GM tree trophic interactions have so far been limited to observations on the evolution of populations of associated species, mostly herbivory insects.

Results from a first study on field trial-grown *4CL* down-regulated transgenic aspen (*P. tremuloides*) with reduced lignins and altered growth showed no effects of the genetic modification on growth and survival of forest tent caterpillars (*Malacosoma disstria*) (Brodeur-Campbell *et al.*, 2006). Effects on feeding performance of gypsy moth (*Lymantria dispar*) larvae were shown to vary among tree transgenic lines, but no potential biochemical alterations in the transgenic lines could be directly linked to *4CL* suppression (Brodeur-Campbell *et al.*, 2006).

Similarly, soil microbial community responses to altered lignin biosynthesis in *4CL* down-regulated and/or *F5H* over-expressing *P. tremuloides* appeared to vary among soils differing by their chemical and physical properties, but results on root biomass, diversity of microbial communities were not consistent, making it difficult to assess any potential ecological impacts (Bradley *et al.*, 2007).

The lignin modifications induced by *CAD* or *COMT* down-regulation in transgenic poplars (*P. tremula x P. alba*) had no unexpected biological or ecological impacts. Interactions with leaf-feeding insects, microbial pathogens and soil organisms were unaltered (Halpin *et al.*, 2007). Likewise, no difference in the diversity of the soil bacterial community could be found (Pilate *et al.*, 2002; Halpin *et al.*, 2007). While a preliminary study has shown that lignin modifications resulted in higher rates of root decomposition than in the wild type (Pilate *et al.*, 2002), it was later demonstrated that only in the short-term, decomposition rate of roots in the soil was slightly increased for the most affected trees, suggesting that the lignin modification led to a decreased ability of modified lignins to protect polysaccharides from microbial attack: this actually occurs during the first steps of decomposition, whereas in the long-term, such differences were no longer apparent (Hopkins *et al.*, 2007). In addition, the

decomposition in soils of sections of trunks from similar transgenic trees did not reveal any significant differences in total CO₂ production or in total mass loss from the trunk material (Tilston *et al.*, 2004). Otherwise, the evaluation of the same transgenic lines, revealed that effects of the different genetic modifications on leaf litter quality, its decomposition rate, and colonization by aquatic insects in three natural Scandinavian streams, were shown to be small compared to variations due to environmental conditions (Axelsson *et al.*, 2010).

Finally, the diversity of fungal communities associated with poplar roots from field-grown transgenic poplar lines with suppressed expression of *CAD*, *COMT* (Pilate *et al.*, 2002) or *CCR* (Leplé *et al.*, 2007) was evaluated by a metagenomic approach using 454-pyrosequencing. It appeared that none of the genetic modifications had any impact on the diversity of ectomycorrhizal fungi. The only significantly lower diversity indices measured for one transgenic line was most likely related to its altered biomass production suggesting a link between plant productivity and fungal diversity (Danielsen *et al.*, 2011).

Feeding preferences and growth performances of common boreal insect herbivores (Lepidoptera and Coleoptera) were evaluated on the leaves of transgenic *COMT* down-regulated silver birch (*Betula pendula* Roth) with lower S/G ratio in their lignins. On average, but not significantly, lowest growth rates for two species were measured when larvae were fed on leaves with G-rich lignins, suggesting a negative effect of the lignin modification on leaf digestibility (Tiimonen *et al.*, 2005). Overall, this study led the authors to conclude that, despite variations of feeding preferences of insect herbivores among the different birch lines, feeding preferences were not directly associated with lignin modification. Although scarce, all these preliminary studies led to the conclusion that lignin modified trees have little or no effect on these trophic interactions.

IV. Synthesis, conclusion and prospects

The results obtained from lignin-modified transgenic trees emphasize the need for running field trials to evaluate in real conditions the effects (sometimes unforeseen) of genetic modifications.

As stated in the corpus reviewed in the previous section, nearly all the genes coding for enzymes from the lignin biosynthetic pathway have been identified, excepting genes coding for proteins involved in monolignol translocation to the extra-cellular matrix before lignin polymerization. Transgenic trees modified for these genes have been generated and evaluated for the effects of their down-regulation or overexpression on lignin metabolism, on wood properties with regards to industrial uses (mostly pulp and paper making and now biofuel production) and finally on tree growth and development. A few recent studies have been targeted to transcription factors known to regulate lignin biosynthesis while a few others not directly targeted to lignin modification resulted in transgenic trees altered in lignin metabolism, and some new approaches still at their infancy aim to produce synthetic lignins specially designed for a given industrial use. Finally, it is worth underlining that there is virtually no report on the production of trees with increased lignin amounts. Likewise, nobody has apparently been successful in producing a conifer tree able to synthesize S units by the integration of adequate angiosperm enzymes to process the different substrates leading to the production of sinapyl alcohol. This strongly suggests that this inability of conifers to produce S units may not be limited just to the absence of the suitable enzymes.

A. Lessons from the past studies

Among all the research efforts invested over these last twenty years, only a rather limited number of target genes gave promising results in the field, i.e. a reduction of lignin content or a modification of the lignin polymer making it more amenable to efficient delignification.

1. It is easy to reduce lignin content but often with negative effect on growth

A number of studies were very successful in producing transgenic trees with different degrees of reduction in lignin content through modulation of lignin biosynthetic enzymes (e.g. C4H, C3H, 4CL, CCoAOMT, CCR, CAD and peroxidase). Such lignin reduction appears most of the time beneficial for pulp and paper production. Nevertheless, field trial evaluation of transgenic trees with reduced lignin contents indicated that beyond a certain level, lignin reduction resulted in major detrimental effects on the growth and development of the trees, inducing an increase in winter mortality and a reduced biomass, as has already been observed for perennial herbaceous species (Casler *et al.*, 2002). The threshold appears to be around a 20% reduction: interestingly, this threshold fits with the estimated natural variation of lignin content measured both within and between tree species (Sederoff *et al.*, 1999). Moreover, it

appears that, often, only the most affected trees that could grow normally in near optimal greenhouse conditions were kept for further small-scale field trial evaluation, leading sometimes to deceiving results in terms of growth in the field. This suggests that in field trial evaluations it would be wiser to keep a larger number of different transgenic lines representing a large array of lignin alterations, in order to be able to identify the degree of lignin alteration interesting for a given use, but still compatible for normal or near-normal tree growth and development: for example, this approach was successful to identify a *CAD*-down-regulated transgenic line, exhibiting both normal growth and wood easier to delignify for paper production (Pilate *et al.*, 2002). This is also the case for a *4CL*-down-regulated line with reduced lignin content that grows at least as well as wild type trees in 3 different sites (Stout, 2011).

It is also worth pointing out that lignification is a highly variable process strongly dependent on both environmental (external) conditions and the physiological (internal) state of the trees: effects of environmental conditions are illustrated by the variations in results obtained from multisite field trials (Stout, 2011, Pilate *et al.*, 2002). Effects of the physiological state of the trees can be easily estimated by tracking in the different parts of the tree the pattern of colored phenotypes generally observed in lignin-modified trees. From published results, it seems that the maximum effect is generally observed at the base of the stem, with eventually a uniform coloration, while this coloration may be weakest or patchy in other parts of the plants. Likewise, it seems that at least in some cases, there is an attenuation of the effects of the transformation over the year or during tree development as observed for example for *COMT* down-regulated poplars, when comparing OMT activity in young and older trees (see van Doorselaere *et al.*, 1995, Pilate *et al.*, 2002). The evolution of the colored phenotype can be a good way to evaluate the strength and stability of the modification that may be different according to the strategy used (antisense, sense cosuppression, RNAi or overexpression).

2. Lignin structure can be modified

The second promising lignin modification concerns the effects on lignin composition in such a way that lignins will be easier to delignify. Most of the time, such beneficial results were successfully obtained by increasing the proportion of S units in the lignin monomers as demonstrated by *F5H* over-expressing transgenic poplars. However, other promising results were obtained thanks to more subtle changes in the lignin polymer, such as the increase in

free phenolic groups obtained in *CAD* down-regulated trees. Interestingly, such qualitative modifications generally do not result in much alteration in tree growth and development.

In addition, until now, most of the studies have been focused on the evaluation of the lignin-modified wood for pulp and paper production. However, preliminary studies indicated promising results with transgenic trees modified for C3H, C4H, F5H (Hinchee, 2011) and also CCoAOMT (van Acker *et al.*, 2011) for biofuel production. Important efforts are devoted to the domestication of trees for biofuel production using biotechnology, as it may be likely that wood better suited to enhance pretreatment efficiency may not be available within the genetic variation available in forest tree natural populations. Due to these important efforts, we may anticipate that a number of other lignin-modified transgenic trees will be evaluated for this application in the near future as indicated by a number of applications in the US, available at the Information Systems for Biotechnology (ISB) website (<http://www.isb.vt.edu/Default.aspxsite>). For example, a field trial is currently running from 2008 to 2014, in Purdue University, with different poplar trees modified for virtually all the enzymes of the lignin biosynthetic pathway (<http://www.isb.vt.edu/getRelDetail.aspx?bp=07-345-103r> & <http://www.isb.vt.edu/getRelDetail.aspx?bp=10-363-113r>).

3. Effects on the environment

Although a number of studies have concerned the development of lignin-modified trees in natural conditions and the technological properties of the modified wood, it is worth stressing that very few studies have been devoted to the evaluation of potential effects of lignin modified trees on their environment. This probably results from the fact that such studies need specific field trial design and also require specific expertise in ecology that is not necessarily easy to mobilize on the subject. Even if limited in number, these environmental studies suggest that lignin-modified trees have no or very limited effects on neighboring environment. This is probably related to the fact that the goal for technological applications is to obtain a wood with somewhat degraded properties for tree physiology, which is unlikely to increase the fitness of the transgenic trees.

B. Some hints for the future

1. Needs for widening transformable species / genotypes

To date, very few genera have been subjected to genetic engineering aiming at lignin modification: *Populus*, *Eucalyptus*, *Picea*, *Larix* and *Pinus*, and, within these genera, only a few species have been successfully transformed: *Pinus radiata*, *Picea abies*, *Larix leptoeuropae*, *Eucalyptus calmadulensis*... For poplar, the array of transformed species is somewhat wider but remains mostly concentrated on aspen species: *P. tremula*, *P. alba*, *P. tremuloides*... and related hybrids, whereas very few cottonwood species and hybrids have been successfully transformed: *P. trichocarpa*, *P. deltoides* x *P. nigra*... Even within these species, transformation procedures have generally been optimized on only one or a very few number of genotypes. For example, a large number of the studies, described in the first part of this paper, were carried out on the same genotype (INRA 717-1B4, *P. tremula* x *P. alba*) as this genotype is rather easy to transform and is freely available to the scientific community (for more information contact pilate@orleans.inra.fr). Generic transformation protocols need to be optimized, that are efficient on a wide number of genotypes within the same species. Transformation of conifers is a different case, as it involves the production of transgenic material upon regeneration of plants through somatic embryogenesis, a technique that seems much easier to extend to a large number of genotypes.

2. Use of suitable promoters

Analyses of the corpus of experiments described above, indicated that it seems preferable to use a promoter conferring a specific expression during the lignification step in differentiating xylem such as *p4CL1* or *pC4H* instead of the single or double pCaMV 35S promoter, potentially conferring a constitutive expression, that is still used in most studies: the use of such specific promoters may reduce the occurrence of pleiotropic effects of the genetic modification on plant growth and development.

3. The right combination

Only a few studies were devoted to the production and evaluation of transgenic trees modified for more than one gene. Although, double transformation will clearly increase the chance of obtaining trees affected in their growth and development, with the knowledge acquired from the analyses of single transformants, this can be anticipated through the judicious choice of

target genes, and enhance beneficial effects. This will probably be the case with, for example, the production of transgenic trees where both a reasonable reduction in lignin content and an increased S/G ratio have been generated. This was probably the goal of producing trees both down-regulated for *4CL* and over-expressing *F5H* genes, but unfortunately, this objective was misled at the start by the promising results obtained on *4CL* down-regulated greenhouse-grown poplar trees, that were not subsequently confirmed in the field (Hu *et al.*, 1999). From our present knowledge, we can propose that both down-regulation of *C4H* or *CAD* and over-expression of *F5H* using xylem-specific promoters would be a promising combination for investigation in transgenic trees.

C. Need for ambitious plans for experimental field trials to identify efficient innovative biotech trees

Finally, it is important to emphasize that performing field test trials, in multisites and with a large number of genetically modified tree lines, are important requirements for the selection of innovative trees.

As illustrated in the first section, most of the studies on lignin-modified trees were, however, performed in controlled and often optimal growth conditions, mainly in greenhouses. Even when more than 700 field trials of GM trees were reported from a search of publicly accessible databases (Walter *et al.* 2010), field trial studies were carried out only for a small subset of lignin-modified transgenic trees and most often on a single site. This is probably for the following reasons:

- (i) For a number of transgenic trees, the effects of modifications were strong enough to give early visible deleterious effects on tree growth and development, even when grown in favorable greenhouse conditions.
- (ii) The majority of these studies were carried out in University laboratories with no expertise, no possibility or even no aim of setting up a field trial, while the majority of the field trials have been set up by a very small number of public institutions or private companies. Generally, only the data generated by the former are publicly available.
- (iii) For the remaining studies, only a small number of field trials, most often small scale and on a single location, have been set up. This is at least partly due to the burden of efforts and costs to comply with regulations enforced for the dissemination of GMO in field trials

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(Viswanath *et al.*, 2011). Indeed, setting a field trial with transgenic plants also requires a major effort to advertise at different levels on the interests of performing such studies, often in front of a part of society hostile to such dissemination experiments. Sometimes, GM opponents have organized the destruction of transgenic field trials and consequently, the early termination of these labor-intensive experiments: regarding transgenic trees, this unacceptable turn of events has occurred mostly in Europe, in 1999 in Jealott's Hill (England) for lignin modified poplars or in 2004 in Punkaharju (Finland) for sterility in birch.

Presently, in the rest of the world, very few transgenic trees have been commercially planted, only virus resistant papaya in Hawaiï and insect resistant poplar trees in China. At the present time, a few biotech companies, such as ArborGen and Futuragen, still invest in biotech trees, mostly but not only, on eucalyptus, and communicate on a foreseen release of GM eucalyptus to be commercially planted within a few years, in US, Brazil, China, South Africa and Australia. For ArborGen, the freeze tolerant transgenic eucalyptus EH1-427 is currently the subject of a petition for deregulation in the US (Hinchee *et al.*, 2011), whereas objectives for lignin modification are targeted to *C4H* down-regulation and *F5H* over-expression (Hinchee, 2011). Futuragen has not yet communicated on their objectives in the area, while the company announced a decision to run several field platforms in different parts of the world with more than 1000 ha of GM eucalyptus.

In conclusion, the results of twenty years of research on transgenic tree field trials have amply demonstrated the necessity to perform such assays, to fully evaluate the effects (wanted or unwanted) of genetic modifications, and at least to keep up to date with developments in this area. It seems to us very important that such evaluations continue to be done by public institutions in order to provide society with fully objective advice on the possibility of using transgenic trees to fulfill the needs of humankind. Several efforts are presently underway to collectively organize the compilation and analysis of the knowledge gained from transgenic tree field trials and to communicate on the interests of pursuing these actions in order to give the stakeholders the knowledge necessary for them to provide adapted regulations: this is the aim, for example, in Europe of the COST (Cooperation in Science and Technology) Action FP0905, entitled "Biosafety of forest transgenic trees: improving the scientific basis for safe tree development and implementation of EU policy directives" (see: <http://www.cost-action-fp0905.eu/>). This issue is even more important now that we foresee the optimization of wood

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production for new uses such as biofuel production may not be fully effective without biotech trees.

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Caption for the photography

The plantation of lignin-modified transgenic trees in a field trial makes possible the evaluation of the effects of lignin modifications on the GM tree growth and development, in comparison to wild-type plants grown in the same natural conditions. Top left: overview of lignin-modified GM poplar trees grown as short rotation coppices in Ardon, France: the trials was set up after obtaining suitable authorizations from the French Ministry of Agriculture in 2007, with a surrounded cleared area to control sucker development. GM tree agronomical performances were monitored under a variety of environmental cues over the seasons within each year: in summer (top right), in the fall (bottom left) and in winter (bottom right) (credit photo: G. Pilate).