Final Technical Report

Project Title: Genetic Augmentation of Syringyl Lignin in Low-lignin Aspen Trees

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EXECUTIVE SUMMARY

As a polysaccharide-encrusting component, lignin is critical to cell wall integrity and plant growth but also hinders recovery of cellulose fibers during the wood pulping process. To improve pulping efficiency, it is highly desirable to genetically modify lignin content and/or structure in pulpwood species to maximize pulp yields with minimal energy consumption and environmental impact. This project aimed to genetically augment the syringyl-to-guaiacyl lignin ratio in low-lignin transgenic aspen in order to produce trees with reduced lignin content, more reactive lignin structures and increased cellulose content. Transgenic aspen trees with reduced lignin content have already been achieved, prior to the start of this project, by antisense down-regulation of a 4-coumarate:coenzyme A ligase gene (Hu et al., 1999 Nature Biotechnol 17: 808-812). The primary objective of this study was to genetically augment syringyl lignin biosynthesis in these low-lignin trees in order to enhance lignin reactivity during chemical pulping. To accomplish this, both aspen and sweetgum genes encoding coniferaldehyde 5-hydroxylase (Osakabe et al., 1999 PNAS 96: 8955-8960) were targeted for over-expression in wildtype or low-lignin aspen under control of either a constitutive or a xylem-specific promoter. A second objective for this project was to develop reliable and cost-effective methods, such as pyrolysis Molecular Beam Mass Spectrometry and NMR, for rapid evaluation of cell wall chemical components of transgenic wood samples. With these high-throughput techniques, we observed increased syringyl-to-guaiacyl lignin ratios in the transgenic wood samples, regardless of the promoter used or gene origin. Our results confirmed that the coniferaldehyde 5-hydroxylase gene is key to syringyl lignin biosynthesis. The outcomes of this research should be readily applicable to other pulpwood species, and promise to bring direct economic and environmental benefits to the pulp and paper industry.

INTRODUCTION

The removal of lignin from wood for papermaking is a chemical- and energy-intensive, and environmentally hostile process. Genetic engineering of pulpwood species with altered lignin characteristics is regarded as one of the most promising technologies available for producing raw materials in a manner that conserves pulping energy and reduces the environmental impact associated with the pulping process. This project sought to genetically manipulate two lignin biosynthetic pathway genes, one regulating the overall lignin pathway flux, and the other controlling specifically the syringyl lignin branchway, in a pulpwood species in order to produce pulping-friendly wood. Aspen was targeted in this study due to its widespread use for pulping in the Great Lakes, and because of the wealth of molecular and genetic engineering tools available for this species from our previous and ongoing research (Bugos et al., 1991; Tsai et al., 1994; Hu et al., 1998, Tsai et al., 1998; Hu et al., 1999; Harding et al., 2002; Kao et al., 2002; Ranjan et al., 2004). Our objectives were two-fold: genetic improvement of wood properties in trees, and development of analytical protocols for fast, reliable and cost-effective evaluation of transgenic wood properties. Commercial tree species with genetically improved traits, such as reduced lignin content, more reactive lignin structure and increased cellulose content would bring direct economic benefits to the pulp and paper industry. The environmental costs associated with pulping could also be minimized with improved pulping efficiency.

BACKGROUND AND LITERATURE REVIEW

Forests are the most valuable renewable resource on earth, providing a wide range of products in the form of lumber, fibers, chemicals and energy for society's needs. As the world's population continues to grow and land availability diminishes, the depletion of natural resources poses a constant threat to global agricultural productivity. Today, however, our understanding of tree growth and wood formation has advanced to the point where designing trees with improved processing characteristics is becoming feasible. This is especially significant for the pulp and paper industry already burdened with the high environmental and economic cost of removing lignin from wood pulp. As a result, there has been a long-standing incentive to understand the molecular regulation of lignin biosynthesis and to develop healthy trees that produce less lignin and/or more extractable lignin to facilitate pulping.

The reaction rate of lignin degradation during chemical pulping of angiosperm species is inversely related to lignin content, but directly proportional to the quantity of the syringyl moiety in lignin (Chiang and Funaoka 1990). Thus, both lignin quantity and structural composition (*i.e.*, syringyl-to-guaiacyl (S/G) monolignol ratio) affect pulping efficiency, and represent two significant traits targeted in forest tree improvement programs. Unlike forage species, such as maize and sorghum, where mutants deficient in enzymes of the lignin biosynthetic pathway have been identified and shown to possess improved nutritive value due to increased stem degradability (*e.g.*, Cherney et al., 1991), lignin-mutant trees are rare. To date, only one lignin-mutant tree (*i.e.*, loblolly pine clone 7-56), deficient in cinnamyl alcohol dehydrogenase (CAD), is known (MacKay et al., 1997). With advances in molecular genetics and biotechnology, it is now possible to introduce low-lignin and high-S/G traits into economically important pulpwood species via genetic engineering. In one such attempt, we successfully generated transgenic aspen (*Populus tremuloides* Michx.) trees with substantially reduced lignin in their stem wood by antisense down-regulation of a lignin-specific 4-coumarate: coenzyme A ligase (4CL1) gene (Hu et al., 1999). The reduced lignin was compensated for by a concomitant increase of cellulose in the stem wood (Hu et al., 1999). To further improve lignin degradability and enhance the value of these trees, we undertook the present study aiming to genetically augment the syringyl lignin moiety in the low-lignin aspen trees.

The pathway leading to the formation of syringyl monolignol in lignifying tissues of angiosperm species has been significantly revised from its "text book" version in recent years (reviewed in Boerjan *et al*., 2003). Available molecular, genetic and biochemical data now support the idea that biosynthesis of guaiacyl and syringyl monolignols shares a common route from phenylalanine to coniferaldehyde, but divides into separate pathways thereafter. For guaiacyl monolignol biosynthesis, coniferaldehye is reduced to coniferyl alcohol in a reaction catalyzed by CAD, but for syringyl monolignol biosynthesis, coniferaldehyde is further hydroxylated and methylated at the C-5 position to give rise to sinapaldehyde which is reduced to the syringyl precursor sinapyl alcohol (Osakabe et al., 1999). The 5-hydroxylation of coniferaldehyde to 5-hydroxyconiferaldehyde is catalyzed by coniferaldehyde 5-hydroxylase (CAld5H), which represents the branch point for diverting guaiacyl lignin pathway intermediates into syringyl monolignol biosynthesis. The CAld5H gene, therefore, regulates the first committed step of the syringyl lignin branchway, and is an ideal target for genetic engineering of monolignol composition. Two previously isolated and biochemically characterized CAld5H genes, *PtCAld5H* and *LsCAld5H* from aspen and sweetgum, respectively (Osakabe et al., 1999), were used in this study for over-expression in the low-lignin aspen trees. The use of both homologous (aspen) and heterologous (sweetgum) transgenes was intended to compare their effects, and to provide an alternative strategy (*i.e.*, heterologous gene) to overcome possible pitfalls associated with homology-dependent sense co-suppression (Tsai et al., 1998).

In anticipation of increased applications of genetic engineering for pulpwood tree improvement, analytical methods for rapid screening of wood chemical properties of transgenic trees will be needed. Traditional wet chemistry methods are time-consuming and often require large (grams) sample sizes. Therefore, a second objective of this study was to employ two high-throughput analytical methods to facilitate phenotypic screening of wood chemical properties, and to provide validation data for their use in transgenic trees. The pyrolysis Molecular Beam Mass Spectrometer (pyMBMS) technique developed in the National Renewable Energy Laboratory and the solid-state ^{13}C-NMR method are particularly suited for this purpose (Tuskan et al., 1999). Both methodologies require only milligrams of sample and can analyze more than 50 samples per day. Their application in lignin structure analysis of genetically engineered aspen trees is presented.

METHODS

Plant Material

Wild-type aspen and two 4CL1 transgenic aspen lines, one with a 10% lignin reduction and the other with a 40% reduction (Hu et al., 1999), were micropropagated by tissue culture (Tsai et al., 1994) and used in this study

Gene Constructs

CAld5H genes from aspen and sweetgum were used to construct binary vectors in a sense orientation, under control of (1) the Cauliflower Mosaic Virus (CaMV) 35S constitutive promoter or (2) the aspen Pt4CL1P promoter, for xylem-specific targeting (Hu et al., 1998; Harding et al., 2002). Since the 4CL1 transgenic aspen already carry a hygromycin B phosphotransferase (HPT) gene for hygromycin resistance, the neomycin phosphotransferase II (NPT II) gene for kanamycin resistance was used as a selectable marker in these constructs.

Aspen Transformation and Characterization

Agrobacterium-mediated transformation was conducted according to Tsai et al. (1994) using leaves from greenhouse-grown aspen plants. Integration of the CAld5H and the NPTII transgenes into the genome of control and 4CL1 transgenic aspen was verified by PCR and Southern hybridization. Transgenic trees approximately 8 months old were harvested to collect developing xylem for CAld5H gene expression and protein analysis according to our established procedures. Lignin content of stem wood was determined by the Klason and acetyl bromide methods (Chiang and Funaoka, 1990) and lignin structure (syringyl to guaiacyl lignin ratio) analyzed by thioacidolysis according to Rolando et al. (1992).

Molecular Beam Mass Spectrometry

Spectral analysis of cell wall components was conducted as described in Tuskan et al. (1999) using extractive-free stem wood meal of control and transgenic aspen. Calibration models were developed using several hybrid poplar lines exhibiting natural variations in stem lignin content and monomeric structure. The model was then employed for estimation of S/G ratios of the transgenic aspen wood samples produced in this study. Multivariate statistical analysis was performed using commercially available software (Unscrambler, SPSS).

RESULTS AND DISCUSSION

Four binary vectors, *35S::LsCAld5H, 35S::PtCAld5H*, *XP::LsCAld5H* and *XP::PtCAld5H* harboring the sweetgum (*LsCAld5H*) and aspen (*PtCAld5H)* genes in a sense orientation under control of either a constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter, or a xylem-specific aspen 4CL1 promoter (Hu et al., 1998; Harding et al., 2002) were constructed (**Figure 1**) for aspen transformation.

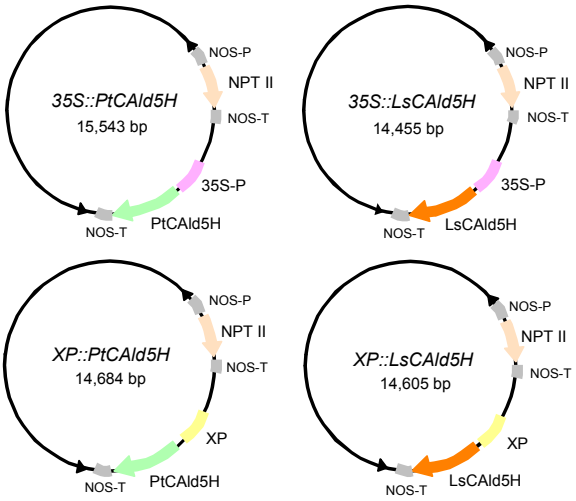

Figure 1. Binary constructs used for aspen transformation. NOS-P, nopaline synthase promoter; NPT II, neomycin phosphotransferase II; NOS-T, nopaline synthase terminator; 35S-P, cauliflower mosaic virus 35S promoter; XP, xylem-specific 4CL1 promoter.

Two low-lignin aspen lines, one with a 10% lignin reduction (referred to as the 10%LR line) and one with a 40% reduction (*i.e.*, the 40%LR line), were selected based on their field growth performance, along with the wildtype aspen control, for transformation with the *CAld5H* constructs. The selected 10% and 40% LR lines were the best performers in a 3-year field trial in northern Michigan, showing a >100% and a ~40% volume growth enhancement, respectively, over the wildtype control. *Agrobacterium*-mediated transformation using young leaves of greenhouse-grown, vegetatively propagated plants was conducted according to Tsai et al. (1994). Regeneration of putative transgenic plants via organogenesis was achieved using kanamycin as a selection agent. Rooted whole plants were transferred to soil media and acclimated to greenhouse conditions. Over 100 independent transgenic lines were generated.

Genomic DNA was extracted from young leaves of greenhouse acclimated transgenic plants. Polymerase chain reaction (PCR) was used to verify the presence of the selectable marker gene using primers flanking a 780 bp NPT II fragment (**Figure 2**). RNA was extracted from de-ribbed young leaves and developing xylem of control and transgenic plants. Northern blot analysis revealed an elevated expression of *35S::CAld5H* in the leaf tissue of transgenic aspen. Up-regulation of *CAld5H* in xylem was more evident in transgenic *35S::PtCAld5H* than in transgenic *35S::LsCAld5H* plants (**Figure 3**). Crude microsomal proteins were extracted from

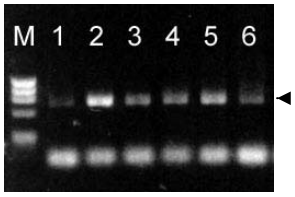

Figure 2. Polymerase chain reaction (PCR) amplification of NPT II fragment (arrow) from 6 representative transgenic lines (1-6). Lane M, DNA molecular markers.

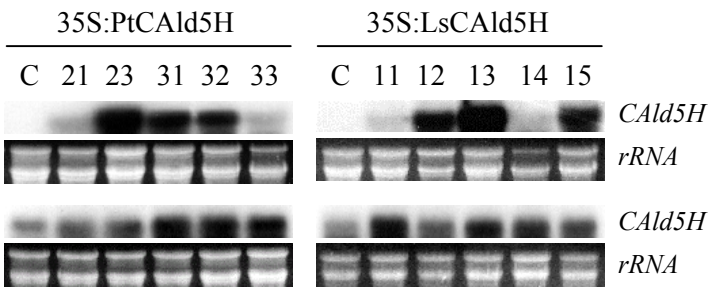

Figure 3. Northern blot analysis of *CAld5H* expression in control and various transgenic lines. Top panels in each group are RNA blots from leaf tissues, and the bottom panels are from xylem. Ethidium bromide-stained rRNAs are shown as loading controls.

de-ribbed young leaves and developing xylem for Western blot analysis. Using antibodies raised against the recombinant LsCAld5H protein (Osakabe *et al.,* 1999), an immuno-signal with expected molecular weight (~58 KD) was detected in leaves of the transgenic *35S::PtCAld5H* plants. An immuno-signal was also detected in xylem microsomal proteins of the transgenics, as well as the control since CAld5H is naturally present in high abundance in lignifying xylem tissue. A slight increase of the immuno-signal intensity was observed for some transgenic lines. The results suggested that both the homologous (*PtCAld5H*) and heterologous (*LsCAld5H*) sense gene constructs, driven by the CaMV 35S promoter, effectively caused over-expression in leaf and xylem tissues of transgenic aspen. Reverse transcription (RT)-PCR has also confirmed up-regulation of *CAld5H* in developing xylem of wildtype and low-lignin transgenic aspen lines harboring *XP::LsCAld5H* or *XP::PtCAld5H*.

Wet chemical analyses of *35S::CAld5H* transgenic aspen wood samples were conducted. Lignin content did not change based on Klason determination, but the lignin S/G ratio was increased by up to 30% in the *35S::LsCAld5H* transgenics and by up to 50% in the *35S::PtCAld5H* transgenic lines based on thioacidolysis analysis. As a next step to correlate wet chemical data with results derived from high-throughput methods, calibration models for rapid analysis of cell wall chemical components by pyMBMS and NMR analyses were established using conventionally bred hybrid poplar and available transgenic aspen wood samples. Projection to Latent Structure (PLS) models were constructed for pyMBMS estimation of stem wood lignin monomeric composition, using 14 different *Populus trichocarpa* × *Populus deltoides* (TD) and 12 different *Populus deltoides* × *Populus nigra* (DN) clones that had an S/G ratio range of 1.1 to 2.6 as measured by thioacidolysis. The PLS model developed had a correlation of 0.94 and root mean squared error of prediction of 0.17. S/G ratios determined by taking the ratio of the summed intensities for peaks assigned to S and G monomers also correlated well with the S/G ratio measurements (r =0.87).

Solid-state, delayed decoupling, ^{13}C-NMR with cross polarization and magic-angle spinning was used to acquire spectra and measure the S/G ratio using the hybrid poplar wood samples described above. The ^{13}C-NMR S/G ratios were determined for all samples and compared to thioacidolysis S/G ratio results. The results indicated that thioacidolysis and ^{13}C-NMR are not statistically different methods for measuring S/G ratios at low S/G ratio values. However, at higher S/G ratios, the results were statistically different within the same confidence interval. The disparity of the results at higher S/G ratios may be due to the preferential release of S monomers in hardwood species that may occur during thioacidolysis. This preferential release may be due to differences in the distribution of S and G monomers within wood tissues and within single tissue cells. This and previous studies show that ^{13}C-NMR provides an alternative, non-destructive method for measuring S/G ratios for hardwood species. The NMR method was also used to determine the S/G ratio of three *35S::CAld5H* transgenic aspens with higher S/G ratios. Wood samples with S/G ≥ 3 are valuable for calibration as they extend the range of S/G ratios beyond those of conventionally bred *Populus* lines mentioned above. The calibration between thioacidolysis and ^{13}C-NMR measurements reveals a coefficient of determination R^2 = 0.8443.

The NMR experiment confirmed that the S/G ratio of the *35S::CAld5H* transgenic aspen lines is higher than the untransformed trees.

Stem wood lignin S/G ratios of transgenic aspen harboring *XP::LsCAld5H* or *XP::PtCAld5H* were then estimated using the pyMBMS calibration model described above. The results, presented graphically in **Figure 4**, suggest that augmented S lignin biosynthesis has been successfully achieved in at least half of the total transgenic population generated: more than 40 transgenic lines exhibited a stem wood S/G ratio greater than 2.5, versus a typical S/G ratio of 2.2 for wildtype aspen stem wood. It appears that homologous (*XP::PtCAld5H*) and heterologous (*XP::LsCAld5H*) gene constructs were both effective for up-regulation of *CAld5H* expression and S lignin biosynthesis. The use of two different promoters (35S constitutive promoter versus xylem-specific promoter) did not seem to have a differential effect on the outcome. More than 15 transgenic lines showed a stem wood S/G ratio of greater than 3, a more than 50% increase compared to the wildtype aspen. The technology is being tested with other pulpwood species in other federally/Agenda 2020 funded projects, and with industrial partners. Future issues to be addressed include pilot-scale pulping analysis, and field and regulatory evaluations to complete the technology assessments.

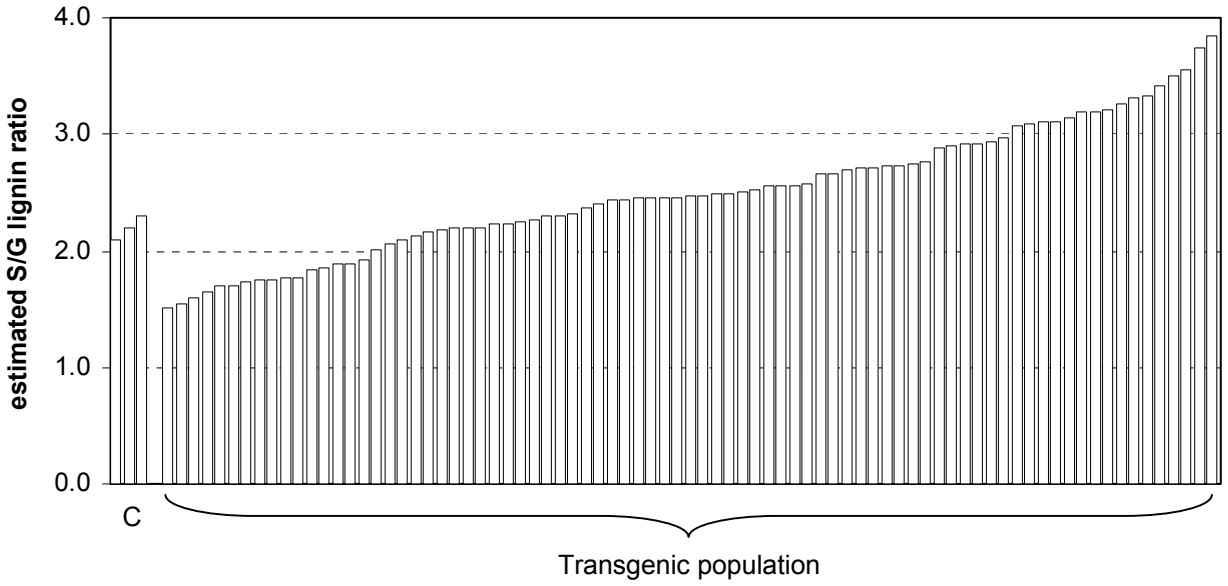

Figure 4. Lignin S/G ratio of control and transgenic aspen estimated by pyMBMS.

CONCLUSIONS

Our results confirm that the CAld5H gene is indeed a suitable molecular target for genetic augmentation of syringyl lignin biosynthesis, and that genetic manipulation of both lignin content and lignin composition in pulpwood species may be achieved via gene stacking. The outcomes of this research should be readily applicable to other pulpwood species, and promise to bring direct economic and environmental benefits to the pulp and paper industry.

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