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Project Title: Improved Wood Properties Through Genetic Manipulation: Engineering of Syringyl Lignin in Softwood Species Through Xylem-Specific Expression of Hardwood Syringyl Monolignol Pathway Genes

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Executive Summary

(1) How the research adds to the understanding of the area investigated: Our long-term goal is to genetically engineer gymnosperms with a lignin that normally found only in angiosperms. Gymnosperms and angiosperms share the same metabolic pathway in the vascular system for the biosynthesis of a type of lignin called guaiacyl (G) lignin. During angiosperm evolution, additional metabolic functions emerged from the G-lignin pathway to biosynthesize a new type of metabolite as a precursor for another type of lignin, called syringyl (S) lignin, for the formation of a final lignin polymer that contains both G and S components. Because gymnosperms have superior fiber quality and angiosperm S lignin can be readily extracted during wood conversion into paper products, the proposed genetic engineer approach would help design strategies for generating desirable wood properties to promote energy efficiency and economic competitiveness of US pulp and paper industry. With respect to the fundamental aspect, the proposed research would lead to greater understanding of the vascular evolution in lignification and adaptation of plants to land. **(2) The technical effectiveness and economic feasibility of the methods or techniques investigated or demonstrated:** We proposed to simultaneously transfer three necessary syringyl lignin specific genes isolated from aspen into a gymnosperm, black spruce, to engineer S lignin in xylem-specific and constitutive manners. The proposed research takes advantage of the established methods and techniques. The effectiveness of genetic transformation of any gymnosperm is still limited. However, the simultaneous transformation of multiple genes and the principle of underlying the proposed approach were technically and economically feasible. **(3) How the project is otherwise of benefit to the public:** The use of the gymnosperm wood with the proposed modification for pulp and paper production is expected to drastically reduce the chemical and energy consumption, and substantially reduce pulp bleaching requirement and thus adverse impacts on the environment. Together, these benefits may allow quantum improvements on energy efficiency, global competitiveness and environmental preservation for the U.S. forest products industry.

Comparison of the Actual Accomplishments with the Goals and Objectives of the Projects:

We had the following specific objectives:

(A) Cloning of spruce xylem-specific promoter

(B) Preparation of aspen *CAld5H*, *AldOMT* and *SAD* gene expression constructs and *Agrobacterium* strains

(C) Transformation of black spruce with aspen *CAld5H*, *AldOMT* and *SAD* gene expression constructs via *Agrobacterium*-mediated multigene transfer and regeneration and propagation of transgenics

(D) Molecular genetic and biochemical characterization of transgenic black spruce trees

(E) S/G protocol establishment

(F) Characterization of lignin and cellulose contents, S/G ratio, and fiber morphology

Outline of Proposed Tasks	Comments
Cloning of spruce xylem-specific promoter	Complete
Preparation of aspen *CAld5H*, *AldOMT* and *SAD* gene expression constructs and *Agrobacterium* strains	Complete
Transformation of black spruce with aspen *CAld5H*, *AldOMT* and *SAD* gene expression constructs via *Agrobacterium*-mediated multigene transfer and regeneration and propagation of transgenics	Complete and see Project Activities Summary on next page
Molecular genetic and biochemical characterization of transgenic black spruce plants	Complete
Lignin content and S/G protocol establishment	Complete
NIR-based characterization of cellulose and xylan contents	Complete
^{13}C NMR quantification of the S/G ratios and other lignin structural details	Complete
All required quarterly reports	Complete
Final Report	This report

Project Activities Summary

We have carried out all tasks as originally proposed and accomplished some additional tasks that were not included in the original proposal.

(1) Original Hypothesis: We used black spruce as the model gymnosperm for the proposed research, because we have established the genetic transformation system for this species. We proposed that S lignin could be engineered in gymnosperm by expressing three aspen S lignin genes, *CAld5H*, *AldOMT* and *SAD*. The proposed project established a model for our industrial partner, ArborGen, to follow for engineering S lignin in loblolly pine, the most predominant pulpwood species in the US.

(2) Approaches Used: Three aspen S lignin specific genes each under the control of a constitutive promoter (35S) were simultaneously transferred into spruce. In parallel, we cloned a spruce xylem-specific (XS) promoter to drive individually the expression of the three S lignin genes in transgenic spruce xylem where lignification occurs. Thus, two types of engineered trees, 35S- and XS-transgenics, were targeted. The importance of having these two engineered tree types is that while XS-trees are anticipated to accumulate S lignin exclusively in xylem tracheid cells, constitutive expression of S lignin genes may, in addition to making S lignin in woody tissues, induce fast growth in transgenic trees as we have observed for transgenic aspen. Our experience with genetic engineering of lignin biosynthesis in trees also predicts that variations in transgene expression levels are bound to occur in these cases. As a result, both transgenic tree types would expect to accumulate lignins with a wide range of S/G ratios, offering a broad spectrum of tree clones to be chosen from for various end uses.

For characterizing wood properties, we developed micro-scale near IR (NIR) based high throughput characterization of wood chemical composition (Fig. 1). This NIR technique has been adapted by many laboratories worldwide for quantification of plant cell wall components.

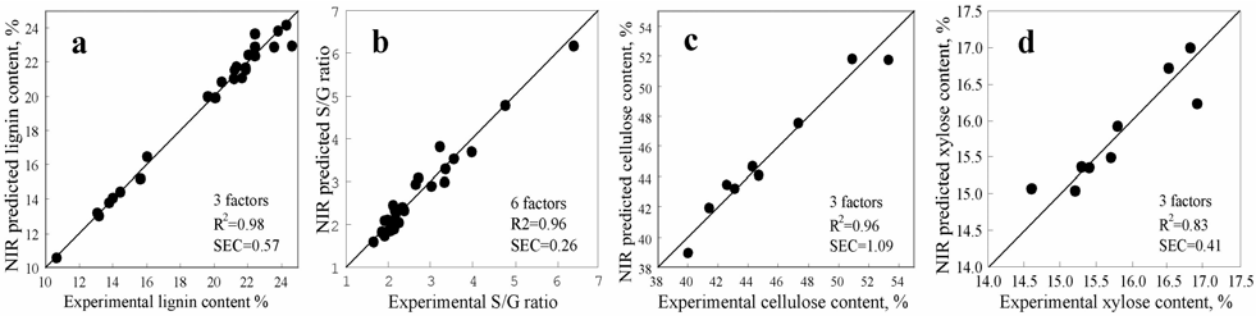

Fig. 1. The correlation between chemically determined and NIR predicted values using woodmeal pellets for (a) lignin (b) S/G ratio (c) cellulose and (d) xylose contents.

We cloned spruce lignin *4CL* gene promoter and demonstrated its function in driving xylem specific gene expression (Fig. 2).

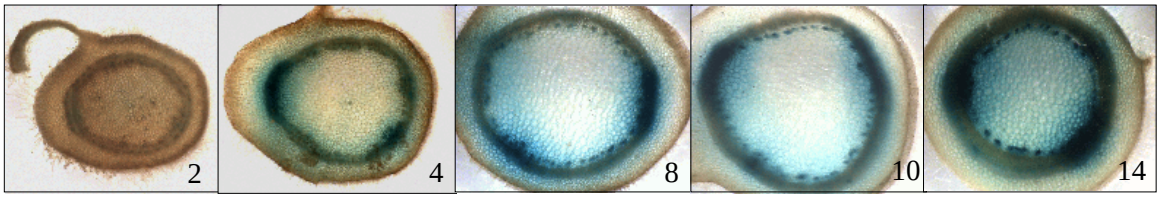

Fig. 2. Spruce *4CL* promoter-driven xylem-tissue specific expression of GUS gene in transgenic tobacco. This promoter activity is specific toward secondary growth, as GUS activity could not be detected in the primary growth stem internal 2 but became intensified in secondary growth tissues (internals 4 and above).

During the course of the project we have developed over 20 distinct spruce callus embryonic lines from over 1,000 zygotic embryos. These callus lines responded differently to callus proliferation, embryo formation and maturation, embryo germination, rooting, and seedling growth. We have selected two best performers, NBS01 and NBS02 lines, for genetic transformation and have generated over 200 transgenic plants.

Seedling number / Gene name	1	2	3	4	5	7	8	9	10
PtCAld5H	√	√	√	√		√		√	√
PtAldOMT		√	√		√				√
PtSAD		√		√			√		√

We have randomly selected transgenics to test gene integration events. As shown in the Table above, we have confirmed positive transformation for 10 transgenic lines.

As expected, some transgenic lines harbored only one of the three S genes, while others contained two of the three S genes. Lines 2 and 10 expressed all three S genes. We have transferred over 200 putative transgenics into soil and maintained in a greenhouse (Fig. 3).

Fig. 3. Transgenic spruce in vitro and in soil.

(3) Problems Encountered: The major difficulty encountered in this project was associated with the spruce genotype we selected for the tasks. The genotype, originated from Canada, was selected when PI Chiang was at Michigan Tech. After, Chiang moved to North Carolina State University, we continued to use this genotype because all preliminary transformation protocols have already been developed. Unexpectedly, all putative transgenics as well as wildtype control perform poorly even in a greenhouse in North Carolina. Not enough woody tissue could be developed for characterization using traditional methods. During the later stage of the project we then started to develop microscale lignin isolation and NMR techniques for lignin structural characterization. We now have established such a characterization system and reported in our last quarterly report.

(4) Summary: Overall we have accomplished all the proposed tasks, including the demonstration of transformation of spruce with three aspen S lignin genes. All transgenics and wildtype plants are still being maintained in our greenhouse. We plan to

apply further funding to conduct detailed lignin structure analysis of these plants using the established NMR techniques.

Products (Publications):

Yamada, T., Yeh, T.-F., Chang, H-m., Li, L., Kadla, J.F., and Chiang, V.L. (2006). Rapid analysis of transgenic trees using transmittance near infrared spectroscopy (NIR). Holzforschung, 60(1): 24-28.

Chiang, V.L., Li, L., Chang, H-m., Kadla, J.F., Sun, J. and Yamada, T. (2004). Genetic engineering of syringyl lignin in spruce. TAPPI Paper Summit, Technical and International Environmental Conference, May 3-5, 2004, Atlanta, GA.

Tsai, C., Zhang, D., and Chiang, V.L. (2004). Genetic Augmentation of Syringyl Lignin in Low-lignin Aspen Trees. TAPPI Paper Summit, Technical and International Environmental Conference, May 3-5, 2004, Atlanta, GA.

Chiang, V.L., Li, L., Chang, H-m., Kadla, J.F., Sun, J. and Yamada, T. (2004). Genetic engineering of syringyl lignin in spruce. 2004 Forest, Wood and Paper Industry Technology Summit, March 28-31, Peachtree City, GA

Chiang, V.L. Lignin biosynthesis in wood formation. Global Biotechnology Forum, March 2-5, 2004, Concepcion, Chile.

Chiang, V.L. Modification of lignin biosynthesis. 2003 Congress on in vitro biology, May 31-June 4, 2003, Portlan, OR.

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