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Significant Increases in Pulping Efficiency in C4H-F5H-Transformed Poplars: Improved Chemical Savings and Reduced Environmental Toxins

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The gene encoding ferulate 5-hydroxylase (F5H) was overexpressed in poplar (*Populus tremula* × *Populus alba*) using the cinnamate-4-hydroxylase (C4H) promoter to drive expression specifically in cells involved in the lignin biosynthetic pathway and was shown to significantly alter the mole percentage of syringyl subunits in the lignin, as determined by thioacidolysis. Analysis of poplar transformed with a C4H-F5H construct demonstrated significant increases in chemical (kraft) pulping efficiency from greenhouse-grown trees. Compared to wild-type wood, decreases of 23 kappa units and increases of >20 ISO brightness units were observed in trees exhibiting high syringyl monomer concentrations. These changes were associated with no significant modification in total lignin content and no observed phenotypic differences. C4H-F5H-transformed trees could increase pulp throughputs at mills by >60% while concurrently decreasing chemicals employed during processing (chemical pulping and bleaching) and, consequently, the amount of deleterious byproducts released into the environment.

KEYWORDS: Ferulate 5-hydroxylase (F5H); lignin biosynthesis; poplar; lignin degradation; syringyl lignin; lignin monomers; pulping; thioacidolysis; DFRC

INTRODUCTION

Over the past decade there has been a radical shift in the way society views forestry. A new paradigm is emerging with an emphasis on conservation of natural forestlands and recognition of the inherent value of trees as carbon sinks, wildlife habitats, scenic vistas, and recreational areas. However, with the growing human population, the world's forests are experiencing increasing pressures to meet demands for wood products, fuel, and agricultural land. With the current global population at approximately six billion people (World Fact Book 2001 http://www.odci.gov/cia/publications/factbook/index.html), the present global timber harvest is ~3.3 billion cubic meters. Assuming that the per capita consumption of timber-based products remains at its current level, it is clear that the predicted growth in global population (>9.4 billion people in the next 50 years) will have a dramatic effect on the required and harvested timber supply.

A large portion of the harvested fiber resource is devoted to pulp and paper production (~160 million metric tons of pulp was produced in 1998). This portion is estimated to increase to nearly 240 million metric tons in the next 50 years. Consequently, a dichotomy is emerging between the need for more wood fiber and the increasing societal pressure to preserve forestland. Trees harvested from intensively managed plantations, or fiber farms, have the potential to alleviate some of these demands, as well as pressures on the natural forests. Currently, tree plantations comprise approximately one-fourth of the global pulp resource, and it has been estimated that this resources will need to increase to ~40% by 2030 to meet future fiber requirements (*1*).

In North America and Europe, poplar (*Populus*) plantations have been initiated because this species is well suited to a northern climate, has a rapid growth rate (~30 m^3/ha/year), and has an inherently lower age of maturity, resulting in shorter rotation times compared to other tree species. Furthermore, biotechnological advances in understanding and manipulating the poplar genome have provided the potential to tailor these trees for specific industrial end uses, such as papermaking.

A significant body of work has focused on altering the expression of every gene in the lignin biosynthetic pathway of

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Arabidopsis, tobacco, and poplar in an effort to alter lignin content and/or chemical ultrastructure (*2*−*4*). The vast majority of research in this area has focused on decreasing lignin content with varying degrees of success (*5*−*9*).

Another approach for the manipulation of lignin for industrial use is the modification of lignin composition. Meyer et al. (*10*) demonstrated that a cinnamate-4-hydroxylase (C4H) promoter used to overexpress ferulate 5-hydroxylase (F5H) eliminates lignin monomer tissue specificity and significantly increases the ratio of syringyl units in *Arabidopsis* lignin. Using the same construct in tobacco and poplar, Franke et al. (*11*) demonstrated similar results, suggesting that F5H is a key enzyme in regulating lignification and controlling lignin monomer composition in woody plants. Recently, two independent studies (*12*, *13*) reported that F5H acts further downstream from ferulate in the lignin biosynthetic pathway, using coniferaldehyde and coniferyl alcohol as substrates. Although Osakabe et al. (*12*) designates the enzyme as coniferaldehyde 5-hydroxylase, we have chosen to use in this paper the conventional and older name, F5H, to denote this gene and enzyme.

This paper reports on the results of experiments aimed at comprehensively evaluating the effects of kraft pulping fiber generated from F5H-altered poplar (*Populus tremula* × *Populus alba*). Our results clearly demonstrate that F5H overexpression significantly improves the kraft-pulping efficiency and does so to an extent substantially greater than any other gene manipulated in the lignin biosynthetic pathway to date.

MATERIALS AND METHODS

Plant Material. The generation of nine lines of C4H-F5H transgenic hybrid poplar (*P. tremula* × *P. alba*) was previously described (*11*), and these, together with wild-type control plants, were maintained as shoot cultures on MS medium with a 16 h photoperiod. Shoots were multiplied from each line by excising nodal segments and allowing axillary buds to elongate. Prior to planting into the greenhouse, 5−8 cm long tips from actively growing shoots were excised and placed on MS medium supplemented with 0.01 μM α-naphthaleneacetic acid (NAA) for 2 weeks to initiate root formation. Shoots were then transplanted directly into potting soil, acclimated for 2 weeks in a high-humidity environment, and grown and transplanted into successively larger pots over the next 2 years.

One-year-old plants were harvested prior to dormancy in the fall. Two-year-old plants were top-pruned at 4 ft above the soil and allowed to overwinter in a nonheated greenhouse. This two-year-old material was then harvested ∼2 months after flushing in the spring. Wood designated as 2 years old was, therefore, the bottom 4 ft section of each 2-year-old tree. Leaves and bark were removed from the harvested stems, and these stems were then left to air-dry at ambient temperatures in the laboratory.

Wood Composition Analysis. Air-dried stems were ground in a Wiley mill to pass a 40-mesh screen and extracted in a Soxhlet apparatus with acetone for 12 h. Lignin content was determined using a modified Klason method derived from TAPPI Standard Method T222 om-98. Briefly, 0.2 g of acetone-extracted ground stem sample was treated with 3 mL of 72% H_2SO_4 for 2 h at 20 °C with mixing every 10 min. This mixture was diluted with 112 mL of deionized water to achieve a final acid concentration of 4% H_2SO_4 and transferred to a serum bottle. The solution was then autoclaved at 121 °C for 1 h and filtered through a medium coarseness sintered glass filter for the gravimetric determination of acid-insoluble lignin. Acid-soluble lignin was quantified by spectrophotometric analysis of the filtrate at 205 nm (Tappi Useful Method UM-250). Carbohydrate concentrations in the hydrolysate were determined by a high-performance liquid chromatograph (HPLC) (Dionex DX-500, Dionex, CA) equipped with an ion exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS 3500 autoinjector (Spectra-Physics, Los Angeles, CA). Prior to injection, samples were filtered through 0.45 μm HV filters (Millipore, Bedford, MA), and a volume of 20 μL was loaded on the column equilibrated with 250 mM NaOH and eluted with deionized water at a flow rate of 1.0 mL min^{-1} followed by a postcolumn addition of 200 mM NaOH at a flow rate of 0.5 mL min^{-1}. Each experiment was run in triplicate.

Monolignol Analysis. The lignin syringyl/guaiacyl (S:G) ratio was determined by thioacidolysis. Extractive-free wood was dried over sodium pentaoxide for 48 h, and 10 mg of the prepared wood was used for thioacidolysis as described by Rolando et al. (*14*), using tetracosane (2 mL of 0.25 mg mL^{-1} in CH_2Cl_2) as the internal standard. The silylation reaction proceeded for a minimum of 1.5 h. All gas chromatography (GC) analyses were performed on an HP 5890 series II, using an HP 6890 series injector and a 15 m × 0.25 mm DB-5 column (J&W Scientific, Folsom, CA). The GC method used a 2.0 μL injection volume, an initial injector temperature of 250 °C, and a detector temperature of 270 °C. The initial oven temperature was 130 °C (held for 3 min) and thereafter ramped at a rate of 3 °C min^{-1} to 260 °C and held for 5 min.

Monolignols were also analyzed using the derivatization followed by reductive cleavage (DFRC) procedure. Fifty milligrams of extractive-free sodium pentaoxide dried wood was prepared using the method previously described by Lu et al. (*15*). All analyses were performed in triplicate.

Wood Density. The specific gravity of each tree was determined according to the standard water displacement method (ASTM Standard Method D2395-93).

Image Analysis. Cell wall thickness, cell lumen area, and ratio of fibers to vessels were determined from transverse sections (18.5 μm thick) of 2-year-old tree stems, stained with safranin and mounted on glass microscope slides. Images were captured on a Meiji microscope fit with a video camera linked to a PC, using Sigma Pro Image Analysis (version 2.0) software. On average, approximately 2300 cells were measured for cell wall thickness and 3200 cells for lumen area.

Chemical (Kraft) Pulping. Laboratory scale kraft pulping was performed using the equivalent of 30 g of oven-dried wood in a 500 mL pressurized reactor in a circulating oil thermostatic bath. Pulping conditions were as follows: 18.3% active alkali, 16% effective alkali, 25% sulfidity, a 10:1 liquor-to-wood ratio, 30 min ramp to 170 °C, which was maintained for 45, 60, or 90 min. The cooked wood chips were then washed extensively with water and blended for 15 min in a standard British disintegrator. The resulting pulp was filtered and washed until the filtrate water was clear. Pulps were then dried overnight at 50 °C prior to weighing for total pulp yield.

Pulp Analysis. kappa numbers (residual lignin) and pulp viscosity were determined using TAPPI Standard Methods T236om-99 and T 230 om-94, respectively.

Pulp Bleaching. Ten grams of pulp was bleached by a standard DED sequence (where D = chlorine dioxide; E = sodium hydroxide). All stages of bleaching were done at 10% consistency. The conditions were as follows: D_0, 0.5% ClO_2, 0.05% NaOH at 80 °C for 78 min; E, 1.3% NaOH at 78 °C for 42 min; D_1, 0.3% ClO_2 at 70 °C for 194 min. Following bleaching, 4 g brightness pads were made and maintained at a controlled temperature (23 °C) and humidity (50%). Brightness measurements were made on a Technibrite Mirco TB-1C.

RESULTS AND DISCUSSION

Increasing Lignin Syringyl Monomer Content Does Not Lead To Undesirable Pleiotropic Phenotypes. Previous attempts to modify lignification in woody plants has led to secondary effects, such as vascular collapse, that may limit the deployment of these technologies in the field. To determine whether F5H overexpression leads to similar undesirable phenotypes, lignin quality and quantity measurements were conducted along with tests to document potential changes in wood cell wall polysaccharide content and vascular development. Thioacidolysis and DFRC are complimentary methods that can effectively be used to determine lignin monomer composition. Both methods demonstrated that lignin in 1-year-

Table 1. Syringyl and Guaiacyl Monomer Contents of Wild-Type and C4H-F5H-Transformed Poplar As Determined by Thioacidolysis and Derivatization Followed by Reductive Cleavage (DFRC)

tree line	total G units[a] (μmol/g)	total S units[a] (μmol/g)	S:G[a]	% mol syringyl lignin	
				thioacidolysis	DFRC
WT	254.4	484.2	1.90	65. 6	64.7
A	178.0	429.9	2.41	70.7	69.8
B	139.7	540.6	3.87	79.5	78.8
C	114.0	538.1	4.72	82.5	81.5
D	110.3	561.0	5.09	83.6	82.7
E	73.9	587.7	7.95	88.8	87.8
F	59.8	492.2	8.21	89.1	88.0
G	74.2	612.6	8.26	89.2	88.8
H	58.9	836.5	14.17	93.4	92.6

[a] Values determined from thioacidolysis analysis. Averages of duplicate experiments (range = 0.05–0.2).

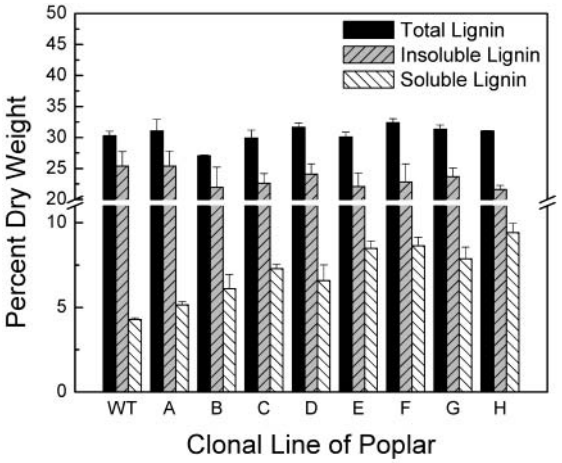

Figure 1. One-year-old poplar wood lignin content versus concentration of mole percent syringyl monomer in wild-type (65.6%) and transgenic lines.

old trees overexpressing F5H was significantly altered in its syringyl/guaiacyl ratio (S:G) when compared to wild type, ranging from 1.9 in the wild-type trees to a maximum of 14.2 in line H (**Table 1**). Our current results demonstrate that the wild type has a ~65 mol % syringyl monomer content and that the transgenic trees range from 71 to 93.5 mol % syringyl, on the basis of thioacidolysis, comparable to the previous results of Franke et al. (*11*), considering that they employed nitrobenzene oxidation (NBO) as their primary mode of monomer determination. These values were supported by monomer quantification by DFRC, which also generally showed a lower syringyl monomer content than NBO (*11*). It should be noted that poplar lignin is known to be esterified with *p*-hydroxybenzoic acid groups (1.5% of total lignocellulosic material), and all lignin analysis to date (thioacidolysis, DFRC, and NBO) cannot account for these moieties in the lignin. Therefore, these analyses serve as a very good comparison between wild-type trees and transformed lines but do not represent absolute syringyl to guaiacyl values. In addition, our results also demonstrate that this relative S:G ratio is maintained in the wood of both years' growth, although it has previously been reported that the S:G ratios of trees can increase over time (*7*, *9*).

Using a modified version of the Klason method, total lignin content of wild-type trees was found to be ~30% of dry weight material, whereas that of the transgenic lines ranged from 27 to 32% (**Figure 1**). Thus, there was no substantial impact of syringyl monomer composition on total lignin; however, a notable trend was observed with regard to the content of

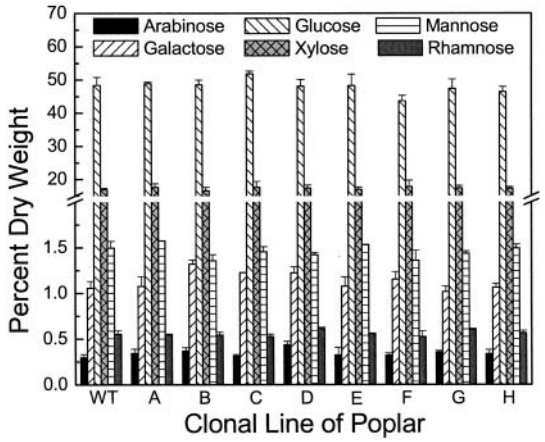

Figure 2. One-year-old poplar wood carbohydrate content versus concentration of mole percent syringyl monomer in wild-type (65.6%) and transgenic lines.

insoluble and soluble lignin. Wild-type trees contained ~25% insoluble lignin, whereas the transgenic lines ranged from 21 to 25%, with a general decrease in insoluble lignin content as mole percent syringyl monomer increased (**Figure 1**). Conversely, there was a significant increase in soluble lignin as syringyl monomer content increased. These results clearly indicate that lignin solubility is positively correlated with syringyl monomer content.

To determine if F5H overexpression has an impact on the polysaccharide portion of the cell wall, a detailed HPLC analysis of total carbohydrates was performed (**Figure 2**). These analyses showed that total carbohydrate content, as well as the ratio of different neutral wood sugars in transgenic lines, was comparable to that of the wild-type. In addition, an analysis of acid sugars (glucuronic and galacturonic acid) indicated that the concentrations of these sugars were also not affected by F5H overexpression (ranging from 0.85 to 0.95% of the original xylem tissue of both wild-type and transformed lines). These results suggest that lignin modification, in this case resulting in >90 mol % syringyl lignin as determined by thioacidolysis, does not impact cell wall polysaccharide deposition.

To investigate the effect of F5H overexpression on xylem cell development, morphological differences between wild-type and transgenic trees were assessed by image analysis of the 2-year-old stem cross sections (data not shown). In these analyses, no significant differences in the ratio of fibers to vessel elements were detected. Additionally, no significant differences between wild-type and transgenic trees were observed in the average fiber lumen area (0.01 mm^2), vessel lumen area (0.5 mm^2), or cell wall thickness (2 μm). Collectively, these results indicate that the xylem in C4H-F5H transgenic poplars develops normally.

F5H Overexpression Has a Dramatic Impact on Kraft Pulping Efficiency. To evaluate the impact of enhanced syringyl monomer content on pulping efficiency, various kraft pulp cooking times (different *H* factors) were used to determine the optimum cooking regimen for effective delignification of the wild-type and transgenic woods. Residual lignin values show significant decreases with increasing mole percent syringyl units in the transgenic lines, under all pulping conditions (**Figure 3**). For example, a kappa value of 36 was attained for wild-type trees pulped at the least severe *H* factor (856), corresponding to a 45 min reaction time, whereas the transgenic line exhibiting 93.5 mol % syringyl achieved a residual kappa of 13 under the

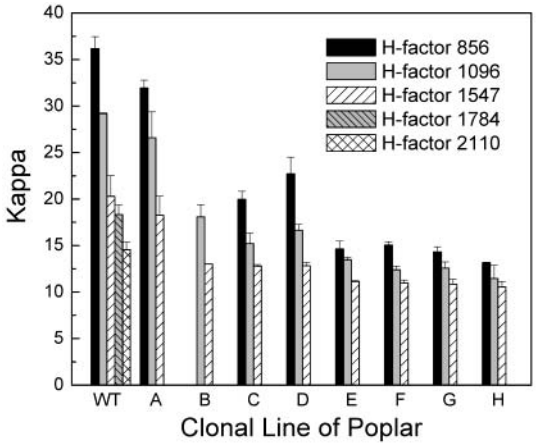

Figure 3. Pulp kappa number versus concentration of mole percent syringyl monomer in wild-type (65.6%) and transgenic one-year-old poplar pulped at different *H* factors. Only the wild-type trees were pulped at the higher (1784 and 2110) *H* factors.

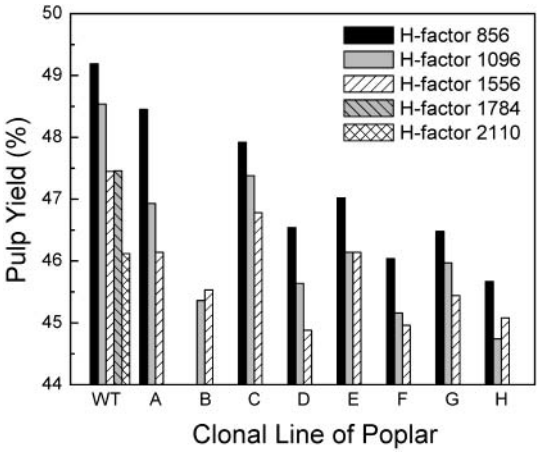

Figure 4. Pulp yield of one-year-old poplar wood processed at different *H* factors. Sample F5H 21 (79.5 mol % S) was not pulped at *H* factor 856 due to limitation in original wood supply. Values represent averages of two pulps.

same conditions, and an *H* factor of 2110 (2.5 h of reaction) was required to achieve a comparable kappa value of 14 in the wild-type.

An evaluation of pulp yield at a given *H* factor (pulping severity) suggests that the transgenic lines have lower yields than the corresponding wild-type pulp (**Figure 4**); however, this decrease corresponds directly to the lower residual lignin content facilitated by more effective lignin removal during kraft pulp cooking. Considering that 6.67 kappa units is equivalent to 1% lignin and using the least severe *H* factor (856) as an example, there is a difference of ~3.5% lignin removed between the wild-type and transgenic line expressing 93.5 mol % syringyl monomers. This difference of 3.5% lignin removed almost completely accounts for the ~3% difference in pulp yield between these two lines. The positive impact of F5H overexpression is further supported by the fact that wild-type trees that were pulped under more severe conditions (*H* factors 1784 and 2110, residual kappa units of ~18 and 14) showed a reduced pulp yield (46.6 and 45.2%, respectively). This indicates that conditions needed to facilitate efficient removal of lignin in the wild type also lead to cellulose degradation, which would consequently compromise yield and strength. The residual lignin and pulp yield trends observed in the 1-year-old trees were also maintained in the 2-year-old trees.

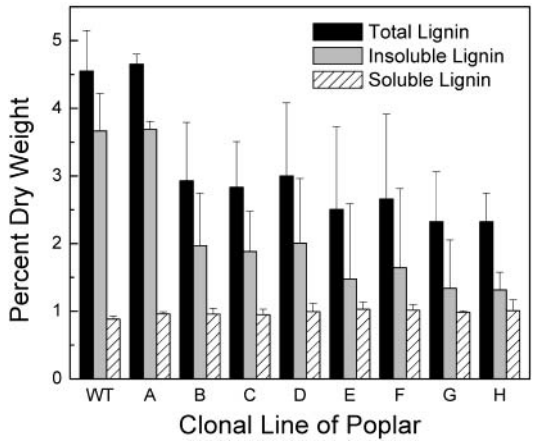

Figure 5. One-year-old poplar pulp lignin content versus concentration of mole percent syringyl monomer in wild-type (65.6%) and transgenic lines processed at *H* factor 1096.

The difference of 23 kappa units between transgenic and wild-type plants is unprecedented in the literature as other research groups have shown only modest reductions in residual lignin, between 4.2 and 5.8 kappa units, using transgenic lines in which lignin biosynthetic genes are down-regulated (*7*−*9*, *16*). These data also suggest that pulping times of trees with increased S:G ratios can be reduced by >60%. Deployment of this technology would thus increase pulp mill capacity while concurrently decreasing chemical costs. It is interesting also to note that trees with >85 mol % S showed no significant decrease in residual lignin with increasing pulping severity (*H* factor 856 compared with *H* factors 1096 and 1556). This suggests that nearly maximum delignification occurred at the lower pulping severities and, therefore, under milder processing conditions.

The klason method was used to determine lignin content of the pulps. As observed in the wood samples, the general trend with regard to pulp lignin is that as the mole concentration of syringyl monomers increases there is a corresponding decrease in total lignin. As an illustration, only the results for samples pulped at *H* factor 1096 are shown (**Figure 5**); however, similar trends were observed with all other pulping conditions (*H* factors). The wild-type pulps have ~3.7% of dry weight as insoluble lignin, and the transgenic samples range from 1.3 to 3.7%. This substantial decrease in insoluble lignin is responsible for the corresponding reduction in total residual lignin observed in the pulp samples, as the concentration of soluble lignin remained constant across all samples (**Figure 5**). These data suggest that the pulping process has removed almost all of the soluble lignin during the early stages of the reactions of pulp digestion and that as the reaction proceeds it reaches a point at which "soluble lignin" removal is limited.

Pulp carbohydrate contents were also determined and compare favorably with kraft pulp carbohydrate contents determined by others (*16*). For clarity, and as they are representative of the other pulps, the results of only *H* factor 1096 are shown (**Figure 6**). Generally, the pulps from transgenic lines have slightly increased carbohydrate concentrations; wild-type has a glucose content of nearly 72%, and transgenic lines range from approximately 73 to 75%, with all of the samples exhibiting ~20% xylose. This increased carbohydrate content in the transgenic lines is to be expected as they have reduced residual lignin contents on a weight basis. The degree of polymerization of the resultant pulp carbohydrates, as determined by pulp viscosity, suggests that the transgenic lines exhibit slightly higher viscosity values (**Table 2**), indicating that the residual pulp

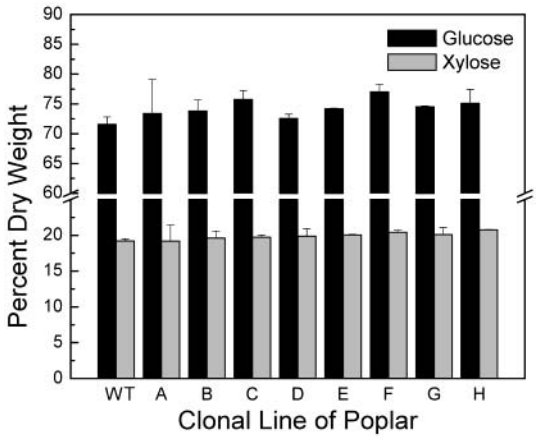

Figure 6. One-year-old poplar pulp carbohydrate content versus concentration of mole percent syringyl monomer in wild-type (65.6%) and transgenic lines processed at *H* factor 1096.

Table 2. Pulp Viscosities[a] of Wild-Type and C4H-F5H-Transformed Poplar Processed at Different *H* Factors

		H factor				
tree line	S:G	2110	1784	1547	1096	856
WT	1.90	36.66	43.42	46.0	42.9	45.1
A	2.41			49.8	56.1	44.9
B	3.87			52.2	50.9	ND
C	4.72			51.4	62.9	60.4
D	5.09			47.2	49.3	52.4
E	7.95			55.0	59.6	66.5
F	8.21			47.6	59.2	64.4
G	8.26			60.1	52.9	57.8
H	14.17			56.9	74.9	66.7

[a] Viscosity values are the average of triplicate experiments and range from 0.12 to 2.8.

carbohydrates are of comparable or larger polymeric length. This implies that the overall strength of the polymer is similar, and not compromised by the F5H overexpression or the processing of this fibrous material, which contains a higher proportion of soluble lignin. These results suggest that paper of equal or better quality can be made from pulp fiber derived from C4H-F5H transgenic trees, particularly because the degree of polymerization of the residual pulp cellulose is higher.

The fibers derived from kraft pulping at similar *H* factors were bleached under similar conditions (DED bleaching) to investigate the effect of the altered S:G ratio on bleaching efficiency and chemical load required to produce white fiber for papermaking. These studies demonstrated pulp produced from transgenic material can be bleached to significantly higher ISO brightness values (**Table 3**). For example, wood digested at *H* factor ~800 had a difference of >30 ISO units between wild-type and transformed lines with high syringyl monomer content. Similarly, at a kraft cook of ~1500 H factor, brightness differences of >20 ISO units were observed. As discussed with the differences in kappa values, these enhanced ISO brightness values are unprecedented in the literature with lignin-modified plants. Other studies that have reported increases in brightness between wild-type and lignin-modified trees have reported value changes of <2 ISO brightness units (*9*). Clearly, these results are related to the higher inherent syringyl content in the lignin matrix, which is more effectively treated by the bleaching chemicals.

Conclusion. It is clear that F5H regulates the ratio of guaiacyl to syringyl subunits in the lignin of plants. By overexpressing F5H in poplar trees it is possible to significantly alter the efficiency for processing wood from such material. Although limited advances have been realized with other genetically modified lines of trees, this paper demonstrates a modification that has far greater implications to pulping than have been reported to date. These data clearly illustrate that C4H-F5H poplars can yield significant savings in pulping process time and chemicals (pulping and bleaching), and the ensuing fiber is likely of equal or better quality. Furthermore, these results build on and surpass previous studies which confirmed that trees specially designed through engineering for "tailored" products, such as pulp and paper, can facilitate a means for significant savings in processing costs. More importantly, the consequence of such results can significantly reduce the ecological footprint left on our environment for such wood processing.

Table 3. Initial and Final ISO Brightness Values of Wild-Type and C4H-F5H-Transformed Poplar Processed at Different *H* Factors

		H factor ~ 800		*H* factor ~1500	
tree line	S:G	initial brightness	DED brightness	initial brightness	DED brightness
WT	1.90	38.5	49.5	43.4	69.1
A	2.41	39.6	50.1	43.5	69.3
B	3.87	43.1	64.1	46.9	77.8
C	4.72	44.3	62.4	45.7	76.3
D	5.09	41.6	63.5	46.0	79.1
E	7.95	45.9	82.2	47.1	91.0
F	8.21	42.2	77.1	44.6	87.1
G	8.26	46.2	73.2	46.9	87.1
H	14.17	44.0	81.1	45.5	89.2

NOTE ADDED AFTER ASAP

Figure 1 was duplicated as Figure 2 in the original ASAP posting of August 23, 2003. This version contains the correct Figure 2.

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