# RNAi-mediated suppression of *p*-coumaroyl-CoA 3′-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism

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***p*-Coumaroyl-CoA 3′-hydroxylase (C3′H) is a cytochrome P450-dependent monooxygenase that catalyzes the 3′-hydroxylation of *p*-coumaroyl shikimate and *p*-coumaroyl quinate. We used RNA interference to generate transgenic hybrid poplar suppressed in C3′H expression and analyzed them with respect to transcript abundance, cell wall structure and chemical composition, and soluble metabolite levels. RT-PCR expression profiles confirmed the down-regulation of C3′H in a number of lines, which generally correlated very well with reduced total cell wall lignin content. The most strongly repressed line was chosen for further analysis and compared with the wild-type trees. In-depth characterization revealed that along with the significant decrease in total lignin content, a significant shift in lignin monomer composition was observed, favoring the generation of *p*-hydroxyphenyl units at the expense of guaiacyl units while the proportion of syringyl moieties remained constant. Suppression of C3′H also resulted in the accumulation of substantial pools of 1-*O*-*p*-coumaroyl-β-D-glucoside and other phenylpropanoid glycosides, and *p*-coumaroyl shikimate, providing further insight into the role of C3′H in the lignin biosynthetic pathway. The data presented indicate that when down-regulated, C3′H becomes a rate-limiting step in lignin biosynthesis and further support the involvement of hydroxycinnamic acid shikimate esters in the lignin biosynthetic pathway.**

phenolic glucosides | syringyl lignin | CYP98 | C3′H

In recent years, most of the genes encoding enzymes specific to the lignin branch of the phenylpropanoid pathway have been cloned and their roles evaluated by using a combination of forward and reverse genetics (1). Lignin itself is a complex, phenolic-based polymer derived from monolignol precursors, which provides structural support, enables water transport, and contributes to plant defense mechanisms against both biotic and abiotic stresses (2). Despite the extensive amount of work that has focused on this important and abundant biopolymer, two key aspects have been elusive: its native three-dimensional structure and operationally effective mechanisms for its removal from the cell wall.

The genes encoding the cytochrome P450-dependent monooxygenases (P450s) cinnamate 4-hydroxylase (C4H) and ferulate 5-hydroxylase (F5H) have been analyzed and evaluated extensively in transgenic plants (3–5). C4H is responsible for the 4-hydroxylation of cinnamic acid (6), whereas F5H hydroxylates the 5 position of ferulic acid, coniferaldehyde, and coniferyl alcohol, although only the latter two are currently considered to be the relevant substrates *in vivo* (7, 8). The nature of the enzyme that catalyzes hydroxylation at the 3 position of the phenolic ring has been the source of long debate; however, several P450s of the CYP98A class were identified recently as coumaroyl shikimate/quinate 3′-hydroxylases (C3′H) involved in cell wall lignification (2, 9).

The role of CYP98A3 in lignification was demonstrated by the characterization of an *Arabidopsis* mutant displaying reduced epidermal fluorescence (*ref*), a consequence of decreased sinapoylmalate accumulation (1). The *ref8* mutant was shown to be defective early in the phenylpropanoid pathway and displayed extremely high levels of *p*-hydroxyphenyl subunits in its lignin, which are a comparatively minor component of the polymer in wild-type plants. The *ref8* mutant is a dwarf, and its xylem is prone to vascular collapse caused by the altered lignin composition, decreased lignin content, or a combination thereof. Furthermore, *ref8* cell walls are more susceptible to the action of polysaccharide hydrolases *in vitro*, and *ref8* plants are vulnerable to fungal attack, suggesting that the cell walls of the mutant may be more readily degradable and/or that products downstream of C3′H may play an important role in disease resistance (2).

The preferred *in vitro* substrates of C3′H are *p*-coumaroyl quinate and *p*-coumaroyl shikimate (6), compounds that until recently had not been considered intermediates in the lignin biosynthetic pathway. In *Arabidopsis*, it is now thought that 4-coumarate-CoA ligase (4CL) activates *p*-coumarate to produce *p*-coumaroyl-CoA, which is subsequently converted to *p*-coumaroyl shikimate and/or *p*-coumaroyl quinate by the action of *p*-hydroxycinnamoyl-CoA:D-quinate/shikimate hydroxycinnamoyltransferase (HCT). C3′H then catalyzes the generation of caffeoyl shikimate and caffeoyl quinate (chlorogenic acid), which can then be converted to caffeoyl-CoA by reversal of the HCT-catalyzed reaction (10). This proposed pathway is supported by the recent cloning and manipulation of HCT from tobacco (10, 11). In summary, the currently accepted model of phenylpropanoid metabolism suggests that during monolignol biosynthesis, hydroxylation at the 3 and 5 positions of hydroxycinnamic acids does not take place at the level of the free acid, as was believed (12).

In *Arabidopsis*, Schoch *et al.* (9) used mRNA and protein localization to demonstrate that the expression of C3′H correlates with the onset of lignification, and plants (*Arabidopsis* and *Medicago*) deficient in C3′H activity have been shown to have significant decreases in lignin content (2, 13, 14). The investigation reported in this work describes the effects of RNAi suppression of C3′H expression in hybrid poplar and further demonstrates the function of C3′H in the production of lignin. In addition, the results show that the impacts of the C3′H perturbation on lignin monomer composition and soluble metabolite pools are substantially different in poplar than those reported in other plant species (i.e., *Arabidopsis* and *Medicago*).

## Results

**Expression of the RNAi-C3′H Construct in Hybrid Poplar.** Suppression of C3′H expression in hybrid poplar (*Populus grandidentata* × *alba*) was achieved by using a ≈350-bp hairpin RNAi construct designed

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**Table 1. Transcript abundance, relative to the control trees, after normalization of transcript abundance to both poplar translation initiation factor 5A and elongation factor 1$\beta$ in control and RNAi-C3′H-transformed poplar**

| Poplar line | C3′H-2 | C3′H-3 |
|---|---|---|
| Control | 1.0 (0.30) | 1.0 (0.30) |
| C3′H-14 | 1.58 (0.47) | 0.05 (0.03) |
| C3′H-21 | 1.21 (0.40) | 0.94 (0.42) |
| C3′H-22 | 1.00 (0.77) | 0.61 (0.24) |
| C3′H-44 | 1.26 (0.61) | 0.84 (0.13) |
| C3′H-53 | 1.04 (0.36) | 0.80 (0.29) |
| C3′H-64 | 1.17 (0.19) | 0.91 (0.54) |
| C3′H-510 | 1.37 (0.60) | 1.14 (0.54) |
| C3′H-515 | 0.85 (0.29) | 0.52 (0.28) |
| C3′H-610 | 0.99 (0.43) | 0.80 (0.19) |

Transcripts of C3′H-1 were not detected in control or transgenic trees. $n$ = 3; standard error in parentheses.

against sequences common to the poplar CYP98A sequences. *Agrobacterium*-mediated transformation yielded numerous independent transformants, several of which did not survive tissue culture propagation, possibly representing individuals in which C3′H expression was strongly suppressed. Among the nine recovered and propagated lines, RT-PCR revealed a substantial but variable reduction in C3′H transcript abundance compared with the wild-type trees (Table 1). Based on the currently available sequence data for poplar (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), three C3′H genes have been identified in the poplar genome. Primers were designed for all three genes, and the associated transcript abundance was determined (Table 1). C3′H-1 transcript was not detected in any line, wild type or transgenic, whereas both C3′H-2 and C3′H-3 were shown to be differentially impacted. The target of the RNAi construct used (C3′H-3) was clearly shown to be significantly down-regulated in several transgenic lines; however, no statistically significant compensatory responses occurred in other members of this gene family in response to C3′H-3 down-regulation (Table 1). Generally, there is a very good agreement with the misregulation of C3′H-3, as measured by transcript abundance, and the overall lignin composition of the transgenic lines of trees.

**Lignin Quantification and Characterization.** All transgenic lines in tissue culture were initially screened for changes in lignin deposition by UV fluorescence microscopy of transverse stem sections (Fig. 1). This initial screen showed that many of the RNAi-C3′H transgenic poplar stems appeared to have impaired lignification. Furthermore, the microscopic evaluation suggested that the structure of the vessel elements of the C3′H-suppressed poplar had been compromised, appearing irregular in shape. Klason lignin (acid-insoluble lignin) analysis of the woody substrate was subsequently used to quantify the extent of lignin deposition. These experiments revealed that the transgenic poplar lines with suppressed C3′H expression ranged in total cell wall lignin content from ≈22.5% (wt/wt) (comparable with control plants) to extreme lows of ≈9% lignin, with several clones displaying intermediate values (Fig. 2). Based on the initial lignin screen and the associated expression data, two transgenic lines were selected for in-depth characterization. These plants include one RNAi-C3′H line with expression and lignin content comparable with that of the wild-type (C3′H-21), and a second line (C3′H-14) that showed the most extreme misregulation, demonstrating very low C3′H transcript abundance and lignin content. After 6 additional months of growth, greenhouse-grown trees of both lines and the corresponding wild-type trees were again evaluated for total cell wall composition, including both total lignin and carbohydrate determination. These tests confirmed the initial screen, showing a lignin content ranging between ≈24% (wild-type poplar line and C3′H-21) and 10.5% (C3′H-14) of tissue dry weight

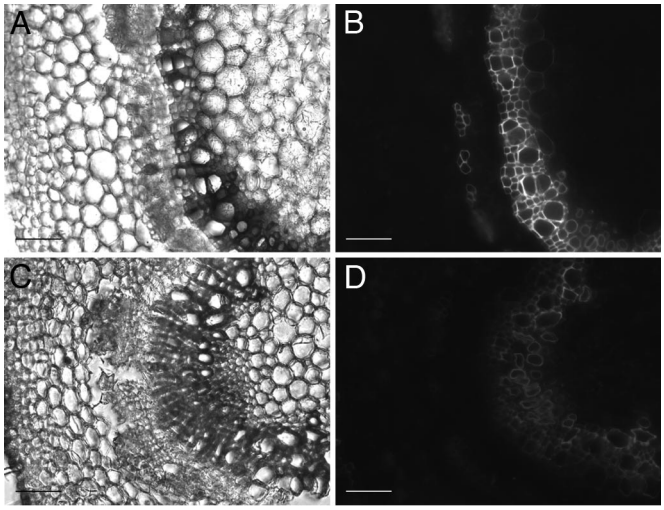


**Fig. 1.** Impact of C3′H suppression on lignin deposition as measured by autofluorescence. Light microscopy of wild-type (*A*) and RNAi-C3′H (*C*), and corresponding UV fluorescence microscopy of wild-type (*B*) and of RNAi-C3′H (*D*) poplar stem cross-sections. (Scale bars: 120 $\mu$m.)

(Table 2). The decrease in lignin content was consistent in both acid-soluble and -insoluble lignin fractions. As a result of the perturbations in lignin deposition, the relative carbohydrate content increased: C3′H-14 trees cell walls consisted of as much as 76% total carbohydrate content by mass compared with wild-type trees that contained only 67% carbohydrate (Table 2). The increased cell wall carbohydrate was accounted for by increases, by weight, in glucose and xylose content without concurrent increases in other cell wall carbohydrates. We ascribe the increased glucose content to more cellulose by weight in the cell wall because the starch content was determined to be comparable with the corresponding wild-type trees (data not shown).

Thioacidolysis was also used to determine the lignin monomer composition of the wild-type and RNAi-C3′H transgenic lines (Table 3). It was apparent that when lignin content was reduced to as low as 16.5% by weight, there was little change in the monomer ratio of the isolated cell wall lignin. However, in line C3′H-14, which displayed the most significant decreases in total lignin content (≈10%), there was a significant reallocation in lignin monomer composition. The released (from thioacidolysis) lignin monomers from this line comprised up to ≈20% *p*-hydroxyphenyl (H) lignin subunits (Table 3). In contrast, the corresponding wild-type and

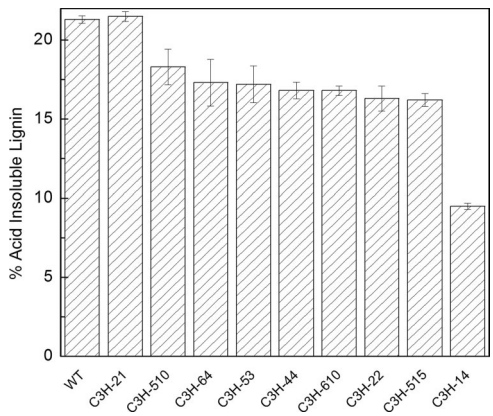


**Fig. 2.** Acid-insoluble lignin content as a percentage of total dry weight as determined by Klason analysis, of wild-type and RNAi-C3′H-suppressed transformed poplar. Mean ± SE were calculated from three plants per line.

**Table 2. Chemical composition of control and RNAi-C3′H-transformed poplar, as determined by Klason analysis**

| | Carbohydrates, mg/100 mg | | | | | | Lignin, mg/100 mg | | |
|---|---|---|---|---|---|---|---|---|---|
| Poplar line | Arabinose | Rhamnose | Galactose | Glucose | Xylose | Mannose | Acid-soluble | Acid-insoluble | Total lignin |
| Control | 0.37 (0.00) | 0.45 (0.01) | 1.09 (0.13) | 45.69 (0.77) | 17.87 (0.48) | 1.76 (0.05) | 2.50 (0.07) | 21.29 (0.25) | 23.78 (0.32) |
| C3′H-14 | 0.42 (0.01) | 0.46 (0.01) | 0.93 (0.03) | 51.10 (0.64) | 21.12 (0.35) | 1.66 (0.03) | 1.02 (0.03) | 9.49 (0.19) | 10.51 (0.19) |
| C3′H-21 | 0.33 (0.00) | 0.40 (0.02) | 0.80 (0.01) | 46.03 (0.71) | 17.30 (0.51) | 1.61 (0.03) | 2.49 (0.04) | 21.54 (0.31) | 21.03 (0.32) |

$n = 5$; standard error in parentheses.

the mildly repressed transgenic line only released trace amounts of *p*-hydroxyphenyl monomers. Surprisingly, the increase in *p*-hydroxyphenyl monomers came at the expense of only guaiacyl (G) lignin subunits, with little apparent change in the relative proportion of the polymer derived from syringyl (S) lignin monomers.

**Pattern of Lignin Deposition.** Consistent with the observed decrease in UV fluorescence and Klason lignin content, histochemical evaluation (Fig. 3) of stem cross-sections after phloroglucinol treatment revealed substantial reductions in staining intensity. Interestingly, the impaired deposition of lignin did not appear to be consistent across the xylem. Whereas wild-type xylem stains relatively uniformly with phloroglucinol, islands of intense staining were observed in the C3′H-down-regulated line, usually associated with groups of vessel elements. Complementary analysis with the Mäule reagent, specific for syringyl units, again gave relatively uniform staining in wild-type sections, although somewhat less staining was associated with the vessel elements, consistent with previous reports of the cell type specificity of lignin monomer composition (15). In contrast, the transgenic line again showed high cell type variation in staining intensity, with little to no staining observed in the vessel elements. These results suggest that these cells deposit either low levels of lignin overall or possibly an H-unit enriched lignin that reacts only to a limited extent with the Mäule reagent.

**Secondary Metabolite Accumulation.** To determine whether down-regulation of C3′H expression had an impact on soluble phenylpropanoid metabolism, reverse-phase HPLC analysis was used to characterize UV-absorbent metabolites in methanolic extracts of wild-type and transgenic stem extracts. Preliminary analyses revealed that wild-type poplar extracts were dominated by six major UV-absorbent peaks, whereas methanolic extracts of the C3′H-14 transgenic line included an array of soluble secondary metabolites not found in the control or weakly repressed line and increased levels of several of the metabolites found in the wild type. In a preliminary attempt to characterize these compounds, the methanolic extracts were saponified, and the hydrolysates were characterized by HPLC. These analyses indicated that saponification released substantial quantities of the *trans*-isomer of *p*-coumaric acid, and minor amounts of the *cis*-isomer (Fig. 4), likely an artifact of processing. These findings indicate that a major component of the metabolites accumulated in the C3′H-14 transgenic line are ester-linked conjugates of *p*-coumaric acid. Liquid chromatography–mass spectrometry (LC-MS) evaluation of the same methanolic extracts revealed that the majority of the *p*-coumaric ester existed as 1-*O*-*p*-coumaroyl-β-D-glucoside and in the form of phenylglucosides common to the phloem of the Salicaceae (16, 17), including populoside, grandidentatin, and trichocarposide. Small amounts of *p*-coumaroyl shikimate (Fig. 5*B*, star) were also shown to be present; however, *p*-coumaroyl quinate, the second potential substrate of C3′H, was not identified (Fig. 5).

**Table 3. Syringyl, guaiacyl, and *p*-hydroxyphenyl monomer composition, and total monomer yield of control and RNAi-C3′H-transformed poplar, as determined by thioacidolysis**

| | Monomer composition, % | | | Total monomer yield, μmol/g dry weight wood |
|---|---|---|---|---|
| Poplar line | H lignin | G lignin | S lignin | |
| Control | 0.2 (0.1) | 35.5 (1.7) | 64.3 (1.8) | 1,592 (90) |
| C3′H-14 | 20.6 (0.8) | 19.3 (1.0) | 60.0 (0.2) | 1,936 (81) |
| C3′H-21 | 0.3 (0.0) | 35.9 (0.4) | 63.9 (0.4) | 1,411 (42) |
| C3′H-22 | 0.2 (0.0) | 34.1 (0.1) | 65.7 (0.1) | 1,707 (186) |
| C3′H-44 | 0.5 (0.2) | 35.2 (1.2) | 64.3 (1.1) | 1,717 (167) |
| C3′H-53 | 0.3 (0.0) | 35.3 (2.4) | 64.4 (2.4) | 1,864 (221) |
| C3′H-64 | 0.3 (0.1) | 34.8 (3.8) | 64.9 (3.7) | 1,714 (86) |
| C3′H-510 | 0.4 (0.1) | 35.2 (2.1) | 64.5 (2.0) | 1,982 (19) |
| C3′H-515 | 0.3 (0.1) | 36.1 (2.7) | 63.6 (2.7) | 1,802 (18) |
| C3′H-610 | 0.3 (0.1) | 35.7 (2.3) | 64.0 (2.4) | 1,827 (289) |

$n = 4$; standard error in parentheses.

## Discussion

This work investigated the effects of the RNAi-mediated suppression of C3′H activity on secondary wall lignification in hybrid poplar. Numerous transgenic lines were generated, with substantial differences in gene expression levels, of which two transgenic lines, representing mild and extreme suppressed lines, were characterized in detail.

Klason analysis of the RNAi-C3′H down-regulated poplar lines revealed significant decreases in lignin content, consistent with the earlier work of Franke *et al.* (1), who observed reductions in the total lignin content of the *Arabidopsis ref8* mutant (20–40% of wild-type levels). Similarly, in *Medicago*, lignin content has recently been shown to decrease from 10 to 7% of total cell wall content in C3′H antisense lines (18). In our experiments, the reduction in lignin content was similar, with a 56% and 59% reduction in acid-insoluble and acid soluble lignin, respectively, which translates into a ≈55% reduction in total lignin in the C3′H-14 transgenic line compared with wild-type. Similar reductions in lignin content (45%) have been reported by Hu *et al.* (19), who used antisense repression of 4CL in aspen (*Populus tremuloides* Michx).

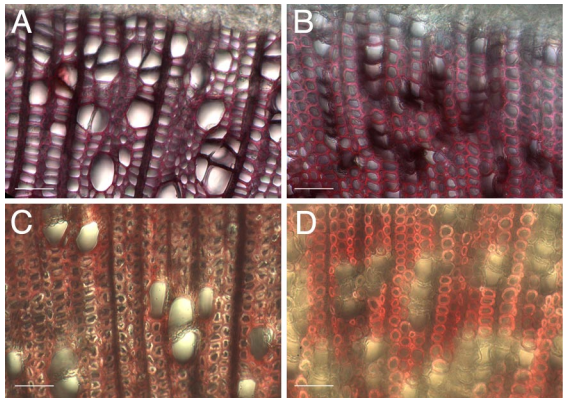


**Fig. 3.** Impact of C3′H suppression on lignin histochemistry. Phloroglucinol staining of wild-type (*A*) and RNAi-C3′H (*B*), and corresponding Mäule histochemical staining of wild-type (*C*) and RNAi-C3′H (*D*) poplar stem sections. (Scale bars: 90 μm.)

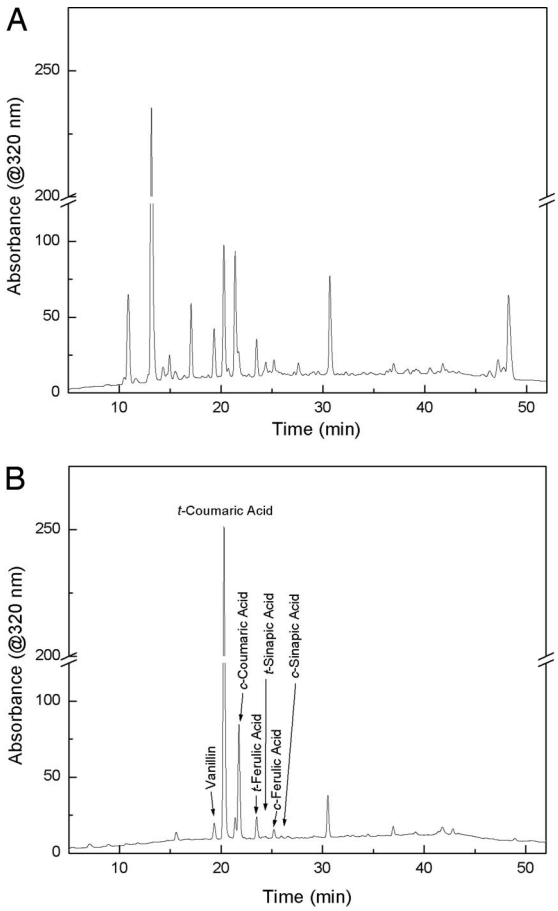


**Fig. 4.** Impact of C3′H suppression on hydroxycinnamic acid-derived metabolites. Saponified methanolic soluble cell wall metabolites of wild-type (*A*) and RNAi-C3′H-14 suppressed transgenic poplar (*B*) as determined by reverse-phase HPLC.

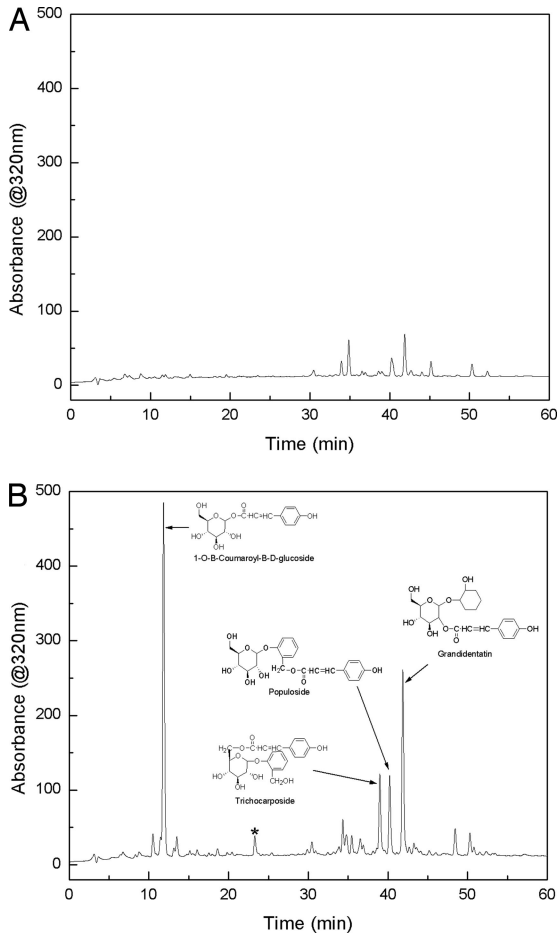


**Fig. 5.** Impact of C3′H suppression on soluble UV-sorbent metabolites. Methanolic extracts of soluble cell wall metabolites of wild-type (*A*) and RNAi-C3′H-14 suppressed transgenic poplar (*B*) was determined by LC-MS. Star, *p*-coumaroyl shikimate.

Histochemical and UV fluorescence microscopy of stem cross-sections support the biochemical evaluation of lignin content; however, the histochemical staining also demonstrated that reduction in lignin content is not consistent across all lignified cell types. This finding is suggestive of spatial and/or temporal misregulation of C3′H and its subsequent impact on lignin deposition. It is possible that this irregular deposition pattern is a function of the efficacy and cell type specificity of the 35S promoter driving the expressing of the RNAi construct, or it may reflect the extent to which RNAi-mediated transcript degradation can be stimulated within specific cell types within the xylem. Employing an alternative, more pathway-specific promoter (e.g., the C4H or 4CL promoters) might achieve a more uniform reduction in cell types within the xylem; however, it may also lead to a more profound suppression of C3′H activity in lignifying cells and more deleterious effect on the xylem lignin deposition, not unlike the severely down-regulated lines generated in this work, most of which did not survive the tissue culture process. These data suggest that for deployment of RNAi-C3′H technology in the field, it might be advantageous to employ a fiber-specific promoter that could decrease lignin content without influencing the integrity of the vessel elements.

The observed reduction in lignin content is consistent with the current model of the lignin biosynthetic pathway. Additional lines, grown to 6 months of age in the greenhouse, analyzed with the Klason lignin procedure, clearly revealed lines with intermediate quantities of lignin, ranging from equivalent with controls to <56% of control values. These results are consistent with the results reported in *Medicago*, where ranges in expression were observed between lines, and parallel alteration in lignin quantity was observed (18).

In the C3′H suppressed line, increases in cell wall carbohydrates of up to ≈13.5% by weight relative to the corresponding wild-type poplar are apparent as a consequence of the reduced lignin content. The increased carbohydrate composition was accounted for primarily by elevated levels of glucose, xylose, and arabinose. The specific increase in these three cell wall carbohydrates simply reflects apparent increases in the arabinoxylan (a major hemicellulose in angiosperms) and cellulose contents of the woody material caused by the altered deposition of lignin. In contrast, slight increases in galactose and mannose were observed in C3′H down-regulated plants, suggesting that there may have been some biologically based compensatory changes in response to decreased lignin deposition. Furthermore, the shift in cell wall composition appears to alter the extractability of the cell walls, as demonstrated by the increased monomer yield in the lines with reduced lignin content. Line C3′H-21 is the only line with lower thioacidolysis yields than the corresponding control trees and is also the transgenic line that has the total lignin content most similar to the wild-type trees. These findings suggest that the higher carbohydrate and lower lignin contents result in a cell wall matrix that is reduced and/or altered in lignin–carbohydrate complexes (LCC) and a lower concentration of insoluble cross-linkages of the noncellulosic polysaccharides. If true, this result would explain why *Arabidopsis*

*ref8* mutant cell walls are more susceptible to the action of polysaccharide-degrading enzymes and more vulnerable to fungal attack (2).

The lignin monomer composition remained consistent among the wild-type and transgenic lines, with the exception of the extremely misregulated line (RNAi-C3′H) where there was a dramatic increase in *p*-hydroxyphenyl moieties (H lignin) and a commensurate reduction in guaiacyl monomers (G lignin). This result is consistent with what has been reported for the *Arabidopsis ref8* mutant in which mutational inactivation of C3′H led to the deposition of a lignin almost solely derived from *p*-hydroxyphenyl moieties (2). Angiosperm lignin is normally dominated by syringyl and guaiacyl units in a common relative ratio of 60:40. Generally, *p*-hydroxyphenyl lignin is insignificant in angiosperms, bordering on nondetectable, but it is important in other species such as grasses and can be detected in the compression wood of gymnosperms. Thus, the current work demonstrates that it is possible to engineer trees with a substantial proportion of *p*-hydroxyphenyl lignin. Recently, similar findings were observed in alfalfa upon down-regulation of C3′H, where *p*-hydroxyphenyl moieties increased from 2.5 to up to 55% (14).

Based on our current understanding of lignin biosynthesis and previous mutational analysis, it is not surprising that an extreme reduction in C3′H activity results in an increase in *p*-hydroxyphenyl monomers. A reduction in the activity of C3′H would be expected to lead to an accumulation of *p*-coumaroyl shikimate and *p*-coumaroyl quinate, the substrates of C3′H and presumably *p*-coumaroyl-CoA, their immediate precursor. Consistent with this hypothesis, *p*-coumaroyl shikimate was identified by LC-MS analyses, at low levels, in stem extracts of C3′H-14 down-regulated trees, but it was not found in wild-type extracts. The diversion of *p*-coumaroyl-CoA directly to cinnamoyl-CoA reductase could easily account for the elevated levels of *p*-hydroxyphenyl subunits identified in the lignin.

In contrast, considering that C3′H catalyzes a step that is common to both G and S subunit synthesis, it would be expected that C3′H down-regulation would lead to a proportional decrease in the incorporation of both monomers into the lignin polymer, as was observed in both the *Arabidopsis ref8* (2) mutant and the C3′H antisense *Medicago* (18). Besseau *et al.* (20) recently showed similar findings when evaluating hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) RNAi-silenced *Arabidopsis* (20), which demonstrated a strong reduction of plant growth, a redirection of the metabolic flux into flavonoids through chalcone synthase, and a highly enriched G subunit lignin composition. Instead, in the current work, the syringyl monomer content in the RNAi-C3′H transgenic poplar was unaffected, and there was a decrease in the percentage of only the guaiacyl monomers. The apparent conflict between these observations could be explained by *in vivo* enzymatic activities that affect the distribution and/or partitioning of carbon to guaiacyl and syringyl monomers in angiosperms, or it may be related to the cell type specificity of the 35S promoter used to down-regulate C3′H expression. The first model would imply that F5H is more abundant or catalytically active relative to cinnamoyl alcohol dehydrogenase and as such maintains constant levels of syringyl lignin monomer synthesis at the expense of guaiacyl monomers when flux through the pathway is reduced. The second model incorporates the observation that lignin monomer content varies in a cell-specific fashion (15) and that the 35S promoter may not drive transgene expression at an equivalent level in all cells. Thus, C3′H expression may be down-regulated more effectively in cells that deposit lignin enriched in guaiacyl subunits, leading to an apparent preferential effect on G lignin biosynthesis. This model is consistent with the differential phloroglucinol and Mäule staining results we observed and with previous observations on the impact of 35S promoter-driven overexpression of F5H in poplar and tobacco (5). Furthermore, this model provides an alternative explanation for differential effects on G and S lignin biosynthesis than the metabolic channeling model that has been put forward by others to explain similar results (21, 22).

The current work employing C3′H-suppressed hybrid poplar further supports the suggestion that *p*-coumaroyl shikimate is the primary natural substrate of C3′H (23, 24). LC-MS analysis did not demonstrate the accumulation of *p*-coumaroyl quinate in the strongly suppressed RNAi-C3′H line but did identify a pool of *p*-coumaroyl shikimate that was not evident in the wild-type trees. Interestingly, down-regulation of C3′H led to a dramatic accumulation of several glucosides of *p*-coumaric acid, including *O*-*p*-coumaroyl-β-D-glucoside and populoside, grandidentatin, and trichocarposide. The last three phenylglucosides have been shown to accumulate in the cortex and phloem of members of the family Salicaceae (16, 17). An evaluation of the tissue-specific accumulation of these compounds revealed that the O-*p*-coumaroyl-β-D-glucoside accumulates in the phloem, leaf tissue, and cambium; whereas populoside, grandidentatin, and trichocarposide are most highly detectable in phloem tissue. This finding suggests that in response to the restricted metabolism of *p*-coumaroyl shikimate, *p*-coumarate is diverted to ester-linked glucosides that may be preventing the potential toxic effects of buildup of this substrate *in planta* and concurrently permitting mobilization to the phloem away from the cambium and developing xylem. Considering that poplar phenylglucosides have been shown to have strong activity against fungi and insect pests (25), C3′H down-regulation and the ensuing accumulation of *p*-coumaric acid-derived glycosides and esters could have several beneficial trade-offs. If this metabolic engineering strategy were used to improve the quality of feedstocks used for biofuel and/or pulp and paper production, it might simultaneously lead to an enhanced metabolic arsenal against biotic pests of poplar, as a consequence of the production of phenylglucosides.

## Materials and Methods

**Construct Development.** An RNAi construct to down-regulate C3′H-3 gene expression in poplar (GenBank accession no. EU391631) was created in the pHannibal vector (26). A C3′H-3 cDNA fragment with introduced BamHI and ClaI restriction enzyme sites was amplified by PCR with oligonucleotides (5′-gtggatccgcgttcaacaacataacaagg-3′ and 5′-gaatcgatgcagtgatcatgtcccaaagg-3′) to facilitate directional cloning into the pHannibal vector. A second set of PCR primers (5′-gtctcgaggcgttcaacaacataacaagg-3′ and 5′-gaggtaccgcagtgatcatgtccaaagg-3′) was used to amplify the same cDNA sequences but with XhoI and KpnI restriction sites to clone the same sequence in the reverse direction to facilitate hairpin formation. Each fragment was then cloned sequentially into the pHannibal vector. The final pHannibal vector containing both C3′H-3 fragments was digested with NotI and cloned into NotI-digested pART27 binary vector (27).

**Plant Transformation.** *P. grandidentata* × *alba* (P39) was transformed with *Agrobacterium tumefaciens* EHA105 (28), employing a standard leaf disk inoculation technique. The binary plasmid (pCC603) was inserted into EHA105, and the bacteria were incubated overnight in liquid woody plant medium (29) with 2% sucrose (WPM) and 100 μM acetosyringone. Leaf disks were cut and cocultured with EHA105 for 1 h, blotted dry, plated onto WPM solidified with 3% (wt/vol) agar and 1.1% (wt/vol) phytagel, and supplemented with 0.1 μM each α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), and thiodiazurone (TDZ) (WPM+NAA/BA/TDZ). After 3 days, the discs were transferred to WPM+NAA/BA/TDZ containing carbenicillin disodium (500 mg/liter) and cefotaxime sodium salt (250 mg/liter). After 3 additional days of selective growth, the discs were transferred to WPM+NAA/BA/TDZ containing carbenicillin, cefotaxime, and kanamycin (25 mg/liter). After two consecutive 5-week periods on this medium, shoot tips were isolated to WPM with no antibiotics.

Plants were confirmed as transgenic by using 35S promoter (5′-gcagctgacgcgtacacaacaag-3′) and poplar CYP98 (C3′H-3)-specific oligonucleotides (5′-caattggggtaccgcagtgatca-3′). PCR amplification was achieved under the following conditions: hot start at 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, followed by 10 min at 72°C.

**Sequencing of Hybrid Poplar (*P. grandidentata* × *alba*) Gene.** Total RNA was isolated from P39 (*P. grandidentata* × *alba*) stem and leaf tissue (30, 31). 5′ RACE (Ambion) was done by using *Pwo* PCR polymerase (Roche) and gene-specific outer primer C3′H-3 GSO (5′-atcatgtcccaaagcagtcc-3′) and inner primer C3′H-3

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GSI (5′-aacggaaacatccaacgaag-3′), from which a 600-bp partial poplar cDNA was obtained. 3′ RACE was used again to confirm the full-length sequence by using C3′H-3 F primer (5′-agacacaatcattggactgctttgggac-3′). RT-PCR was then used to confirm the entire C3′H-3 cDNA sequence by using forward (5′-tctccacaatgaatctccttctg-3′) and reverse primers (5′-tcagtcctcacttttgacagag-3′) after synthesizing cDNA with the SuperScript II first-strand synthesis system (Invitrogen). The resulting PCR products were cloned into a PCR-Blunt cloning vector (Invitrogen) for sequence analysis.

**Transcript Abundance.** RNA was isolated from 100 mg of liquid nitrogen ground samples of stem tissue of plants by using TRIzol reagent (Gibco) according to the manufacturer's instructions. After extraction, 10 $\mu$g of total RNA was treated with 10 units of DNase I (Fermentas) in 6 mM $MgCl_2$. The reaction was incubated at 37°C for 30 min and heat-inactivated at 80°C for 10 min, and then RNA was isolated as per standard protocol. The RNA pellet was resuspended in 40 $\mu$l of RNase-free water.

Equal quantities of RNA (1 $\mu$g) were used for the synthesis of cDNA with SuperScript II reverse transcriptase (Invitrogen) and $(dT)_{16}$ primers according to the manufacturer's instructions. Samples were run in triplicate with Brilliant SYBR Green QPCR master mix (Stratagene) on an Mx3000p real-time PCR system (Stratagene). The primers used for the real-time PCR analysis were C3′H-2 RTF (5′-cggatggcttcagatatgtg-3′) and C3′H-2 RTR (5′-tccaaaccataggcatacaa-3′), and C3′H-3 RTF (5′-ttaggctttggattgtgaac-3′) and C3′H-3 RTR (5′-cattgaaattgatagaagaagtgt-3′). Conditions for all of the PCRs were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. Transcript levels were determined based on changes in $C_t$ values relative to translation initiation factor 5A (32) and elongation factor 1$\beta$ (33), using the following primer sets: 5AF (5′-gacggtattttagctatggaattg-3′) and 5AR (5′-ctgataacacaagttccctgc-3′) and EF1$\beta$ F (5′-ggcattaagttttgtcggtctg-3′) and EF1$\beta$ R (5′-gcggttcatcatttcatctgg-3′), respectively, and compared with the relative transcript abundance of the wild-type trees.

**Wood Compositional Analysis.** Greenhouse-grown plant stem material was ground with a Wiley mill to pass through a 40-mesh screen and then soxhlet extracted with acetone for 24 h. The extractive-free material was used for all further analyses. Lignin content was determined with a modified Klason, where extracted ground stem tissue (0.2 g) was treated with 3 ml of 72% $H_2SO_4$ according to Coleman *et al.* (34). Carbohydrate concentrations in the hydrolysate were determined by using high-performance liquid chromatography (HPLC) (DX-500; Dionex) equipped with an ion exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS 3500 autoinjector (Spectra-Physics). Each experiment was run in duplicate.

**Monolignol Analysis.** Ten milligrams of extractive-free wood was used to determine the monolignol composition by thioacidolysis (35), with tetracosane (2 ml of 0.25 mg/ml in $CH_2Cl_2$) as the internal standard. Gas chromatography (GC) analyses were performed on a ThermoFinnigan Trace GC-PolarisQ equipped with a DB-5 column. The GC method used a 2-$\mu$l injection volume (1:50 split ratio), 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 then ramped at a rate of 3°C per min to 260°C and held for 5 min.

**Microscopy.** Poplar stems were first hand-sectioned with a double-edged razor blade. The samples were then fixed in formalin, acetic acid, and alcohol, dehydrated through an acetone and xylene series, and finally embedded in paraffin. Sections (10 $\mu$m) were then cut from the embedded stem tissue with a microtome, mounted on glass slides, and visualized with a Leica microscope under UV fluorescence.

Histochemical examination of lignin in the poplar stems was analyzed by using both phloroglucinol and Mäule staining. For Mäule staining, stems sections were treated with 5% $KMnO_4$ for 10 min, rinsed with water, and subjected to 10% HCl for 5 min. Sections were rinsed with water and then mounted in concentrated $NH_4OH$ and viewed under bright-field illumination with a Leica DMR with a QICAM CCD camera (Q-imaging). Phloroglucinol staining was achieved by mounting stem sections in a saturated solution of phloroglucinol in 20% HCl. Samples were viewed under dark-field illumination on the Leica DMR microscope 5 min after application of stain.

**Metabolite Analysis.** Poplar stem tissue was ground under liquid nitrogen in a mortar and pestle. Ground tissue (30 mg) was then extracted with 1.5 ml of methanol/water/HCl (48.5:48.5:1) for 4 h at 50°C and then centrifuged for 10 min at 15,000 $\times$ *g*. The supernatant was then removed from the pellet and divided equally into two aliquots. One aliquot was subject to saponification with 1 M NaOH for 16 h at room temperature to facilitate the release of esterified phenolics. Distilled water (1 ml) was then added to each aliquot (methanolic extract and the saponified methanolic extract) followed by an equal volume of ethyl ether. The samples were then mixed thoroughly and allowed to phase partition. The upper phase was then removed and retained, while the extract was again extracted with a second volume of ethyl ether, removed, and pooled. The ether phase was then dried *in vacuo*, resuspended in methanol, and analyzed by HPLC. A Dionex Summit HPLC system fitted with a reverse-phase 0.2-mm $\times$ 150-mm Pursuit column (Waters 5-$\mu$m particle size) autosampler, and a photodiode array detector was used for methanolic metabolite profiling. The methanolic extracts were eluted from the column with a linear gradient of 100% A to 80% B over 60 min followed by a 10-min wash with 100% B, and finally reacclimated with 100% A for 10 min, where eluant A is 5% acetic acid and eluant B is a 75:25 mix of 20% acetic acid and acetonitrile. The flow rate for analysis was 0.2 ml $min^{-1}$, column temperature was 45°C, and detection was at 320 nm.

Detection and identification of phenylglucosides were achieved by LC–MS on a Hewlett–Packard (HP) 1100 LC-MSD-Trap XCT plus. The methanolic extracts were separated on a SB C-18 Zorbax rapid resolution 4.6-mm $\times$ 150-mm 3.5-$\mu$m column at 40°C at a flow rate of 1 ml $min^{-1}$. Separation was achieved by a linear gradient of 95% A, 5% B to 75% A, 30% B over 37 min, where solvent A is water with 0.2% formic acid and solvent B is acetonitrile with 0.2% formic acid. Detection was monitored with a HP 1100 photodiode array detector, and mass determination was achieved by ESI in negative ion polarity.

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