Syringyl lignin production in conifers: Proof of concept in a Pine tracheary element system

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Conifers (softwoods) naturally lack syringyl units in their lignins, rendering lignocellulosic materials from such species more difficult to process than syringyl-rich hardwood species. Using a transformable Pinus radiata tracheary element (TE) system as an experimental platform, we investigated whether metabolic engineering can be used to create syringyl lignin in conifers. Pyrolysis-GC/MS and 2D-NMR analysis of P. radiata TE cultures transformed to express ferulate 5-hydroxylase (F5H) and caffeic acid O-methyltransferase (COMT) from Liquidambar styraciflua confirmed the production and incorporation of sinapyl alcohol into the lignin polymer. Transformation with F5H was sufficient for the production of syringyl lignin in TEs, but cotransformation with COMT improved its formation. In addition, lower levels of the pathway intermediate 5-hydroxycinnamyl alcohol were evidenced in cotransformation experiments, indicating that the introduction of the COMT overcame the inefficiency of the native pine methyltransferases for supporting sinapyl alcohol production. Our results provide the proof of concept that it is possible to generate a lignin polymer that contains syringyl units in softwood species such as P. radiata, suggesting that it might be possible to retain the outstanding fiber properties of softwoods while imbuing them with the lignin characteristics of hardwoods that are more favorable for industrial processing.

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Significance

This study shows that metabolic engineering can be used to imbue pine tracheary elements with an ability to synthesize sinapyl alcohol, a lignin monomer not normally used for lignification in conifers such as pine. The dynamic nature of the lignification process enables pines to incorporate this monolignol, allowing them to produce hardwood-like lignins that are known to facilitate refining processes such as biofuel production and chemical pulping. The potential to improve the refining of conifer-derived biomass through lignin manipulations is important, as even small improvements in yield can lead to significant environmental and economic benefits in such processes.

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Lignin is one of the most abundant terrestrial biopolymers and a major component of both softwoods and hardwoods. It is a heterogeneous cell wall polymer derived primarily from hydroxycinnamyl alcohols via combinatorial radical coupling reactions (1). Importantly, lignin content, composition, and structure affect the processability of woody biomass and have consequently been studied in great detail. Conifers such as pine, spruce, and fir dominate vast areas of land and are consequently of significant ecological and economic value. Conifer wood, often referred to as softwood, has traditionally been used for the production of timber as well as superior strength pulp and paper, but can also serve as a feedstock for bioenergy and biofuels, where its high proportions of hemicellulosic 6-carbon sugars is a significant benefit. Hardwoods are angiosperm trees that differ physically and chemically from softwoods, and have the advantage that the wood is generally easier to pulp or to pretreat to enhance the enzymatic saccharification of the wood polysaccharides to produce sugars for fermentation to liquid fuels, for example.

Conifers have, compared with most hardwoods, a high lignin content that can reach levels of more than 35% (wt/wt) in compression wood, a specialized “reaction wood” made by conifers on the underside of leaning branches or stems for righting growth (2). Conifer lignin consists primarily of guaiacyl (G) units derived from coniferyl alcohol (CA) and lacks hardwoods’ syringyl (S) units derived from sinapyl alcohol (SA) (2). Coniferyl alcohol polymerization generates a more condensed polymer containing higher levels of carbon-carbon linkages between monomer-derived units (SI Appendix, Fig. S1), which, combined with the high lignin content, negatively impacts efforts to refine lignocellulosic materials. Gymnosperms are evolutionarily more primitive than angiosperms, but plants, such as Selaginella, a lycophyte, predating both, has S/G lignins in its stem cortex (3). Although some special lineages of gymnosperms also contain S/G lignins (4), the common softwoods do not, and nor to they possess the genes presumed to be required for the biosynthesis of SA.

Reducing the lignin content in conifers is unlikely to be a viable option to improve the processing of softwoods, as it can compromise plant fitness by causing the collapse of tracheids, the principal building block structures of conifer wood (5). Changing the monomeric lignin composition, however, is seen as a promising alternative (6). Recombinant experiments in pine have already shown that nontraditional monolignols such as cinnamyl alcohol and ferulate can be incorporated into the lignin polymer (7, 8), demonstrating a similar metabolic malleability of lignification to that noted previously in dicots (1, 9–12). Based on these results, we speculated that it might also be possible to incorporate the nonnative, additionally methoxylated, monolignol sinapyl alcohol (Fig. 1) into pine lignin, imbuing softwoods with biomass processability similar to that of hardwoods.

S-rich lignins in angiosperm species facilitate processing of lignocellulosic biomass and thereby provide a key advantage over the G-rich lignins typical of conifers. Lignins rich in S units have a lower degree of condensation, are less complex in structure (1), have a smaller polymer size, and contain higher levels of β-ethers, the easiest of the interunit linkages to cleave using alkali pulping or acidolytic methods. Such lignins are consequently more easily removed from cell wall polysaccharides (13–15). These structural features of S lignins also explain why increasing...
their proportion in angiosperms significantly improves pulping and biofuel production (13–15).

In angiosperms, wood fibers are naturally rich in S units whereas vessel elements contain predominantly G lignin (16). Importantly, recombinant lignin studies in arabidopsis (Arabidopsis thaliana), tobacco (Nicotiana tabacum), and poplar (Populus tremula × alba in this case) have proven that it is possible to increase S-type units to over 90% without compromising plant fitness or performance (13, 14, 17–19). These observations indicate that water-conducting vessel elements with lignin rich in S units are not compromised in their function. We therefore speculated that pine tracheids might also tolerate the incorporation of S units without their function being compromised.

Production of S units in conifer lignins requires at a minimum the introduction of two enzymes (Fig. 1): ferulate 5-hydroxylase ([F5H] also known as coniferaldehyde 5-hydroxylase (CAld5H) to better reflect the now-accepted substrate (20, 21)) and caffeic acid O-methyl transferase ([COMT] also known as 5-hydroxyconiferaldehyde O-methyltransferase (AldOMT) (22)). Neither protein exists in conifers (23), but both are essential for SA biosynthesis in angiosperms (20). However, these might not be the only enzymes involved in the formation of S units in angiosperm lignins. A cinnamyl alcohol dehydrogenase ([CAD]) associated with SA biosynthesis has been identified in arboridopsis (24), and a lignin-related sinapyl alcohol dehydrogenase ([SAD]) that converts sinapaldehyde to sinapyl alcohol was isolated from Populus tremuloides (25). S-specific peroxidases have been identified in a number of angiosperm species (26). These observations, in addition to the fact that monolignol transporters, if they are required, have only been tentatively identified (see below), could add complexity to metabolic engineering experiments designed to introduce S units into conifer lignins.

Transformable callus cultures capable of producing significant levels of differentiating tracheary elements (TEs, SI Appendix, Fig. S4) provide an excellent model system for functional genomics studies targeting secondary cell wall biosynthesis (6). We have developed such a system for radiata pine (Pinus radiata) that produces differentiated TEs with secondary cell walls similar in chemical composition to those of wood tracheids (27). The usefulness of this system for the investigation of lignin biosynthesis in conifers has already been established (6–8, 28, 29).

In this study, we used the transformable P. radiata TE platform to explore the possibility of establishing the production of S lignin units in conifers by metabolic engineering of the phenylpropanoid pathway. Our results provide the proof of principle that it is possible to generate a hardwood-like lignin by imbibing softwoods with the ability to biosynthesize the S monomer, SA.

Results

Generation and Screening of Transgenic Lines. Nondifferentiated P. radiata callus cultures were transformed with vector(s) for F5H with a ubiquitin universal promoter, COMT with a 4CL promoter, and also the SAD gene from Populus tremuloides driven by the ubiquitin promoter, as detailed in Table 1 and SI Appendix, Fig. S2, and as described earlier (28). A minimum of 26 transgenic lines was generated in all three transformation experiments (Table 1). All transgenic lines were transferred to differentiation medium (27) to stimulate the generation of TEs. Differentiated TEs were enriched according to a protocol described earlier (29) and screened by pyrolysis-GC/MS for the signatures of S lignin (30).

Analysis of the resulting pyrograms revealed the transgenic lines that had signatures for S lignin units. The most abundant S-specific pyrolysis products identified were syringol (m/z 154), 4-vinyl-syringol (m/z 180), and 4-propenyl-syringol (m/z 194) (SI Appendix, Fig. S5). pyrolysis products also prominent in pyrograms from angiosperms (30). The pyrolysis-derived S/G ratios in transgenic lines containing S units varied between 0.01 and 0.18 (Table 1 and SI Appendix, Table S2). Transformation with F5H resulted in lower S/G ratios than in the cotransformation experiments that included COMT and/or SAD (Table 1), indicating that the expressed enzymes from these two genes contributed to the generation of S units in pine lignin.

Expression Pattern of Recombinant Genes During TE Differentiation. The expression patterns of F5H, COMT, and SAD were analyzed by quantitative RT-PCR at various stages of the TE differentiation process in two of the F5H/COMT/SAD lines (Line 6 and Line 19) that had the highest S/G ratios in pyrolysis-GC/MS experiments. The expression levels of endogenous P. radiata constitutive ([Ubq]) and lignin-related genes ([C4H, 4CL, HCT, CCoAOMT, CCR, and CAD]) were assessed in parallel for reference. These experiments revealed good expression levels for F5H throughout the differentiation process in both lines, mirroring the expression of the Ubq gene (SI Appendix, Fig. S3). Expression levels for SAD were, despite using the same promoter as for F5H, several orders of magnitude lower (SI Appendix, Fig. S3). COMT expression followed the trend of the endogenous lignin-related genes in both lines, reflecting the value of using of the Pt4CL-promoter (19) in this construct (SI Appendix, Fig. S3).

Chemical Analysis of TE Cultures. Pyrolysis-GC/MS. The potential to sample callus cultures at different stages of TE differentiation combined with the ability of pyrolysis-GC/MS to analyze minute amounts of lignin was used...
to investigate whether S/G ratios changed during TE differentiation in transgenic lines 6 and 19. Analysis of material from each line collected at two-day intervals revealed high S/G ratios at the beginning of the differentiation process that declined during TE differentiation (Fig. 2 and SI Appendix, Table S3).

Pyrograms of purified TEs from all transgenic lines containing S lignin were also analyzed for the presence of diagnostic products from pathway intermediaries (Fig. 1) such as 5-hydroxyvinyl-guaiaicol (SOH-VG; m/z 166, SI Appendix, Fig. S5); SOH-VG was released only from transgenics containing S lignin and not from wild-type controls. The ratio of SOH-VG to the corresponding S lignin product, vinyl-syringol (VS), ranged between 0.8 and 4.0 in lines transformed with FSH only, and between 0.1 and 0.4 in cotransformed lines. The most obvious interpretation for this result is that COMT contributed to the biosynthesis of S lignin and that O-methylation was otherwise limited, as is logical from the biosynthetic pathway depicted in Fig. 1.

Two-dimensional-NMR spectroscopy. NMR experiments are capable of identifying S-containing units in structures that unambiguously establish SA’s role as a monomer in the lignification. More detail on the cell wall structures in the transgenic FSH/COMT/SAD lines 6 and 19 and a wild-type control were deduced from 2D-NMR. For in-depth analysis of lignins, enzyme lignins (ELs) were isolated from TEs via digestion with crude cellulases, leaving all of the lignin and residual polysaccharides (7, 8). The ELs were swelled in dimethyl sulfoxide-d6/pyridine-d5 (4:1, vol/vol) (31, 32) and subjected to 2D short-range 1H–13C correlation (HSQC) experiments. In the aromatic regions of the HSQC spectra of ELs from the transgenic lines (Fig. 3 A and B), S aromatic signals (S2,a) were clearly observed along with predominant G aromatic signals (G3 and G4,a) whereas, as expected, no S aromatic signals were observed from the wild-type control (Fig. 3C). Volume integrations estimated that S units accounted for 8% and 6% of the total lignin aromatics detected in these lines. Production of S units via radical coupling of SA in the transformed lines was further evident from the new signals arising from S-type β-aryl ethers (i.e., β-syringyl ethers, 1) in the aliphatic side-chain regions in which the diagnostic correlations for the various lignin interunit linkage types are resolved. Close comparison of these spectra indicated that incorporation of sinapyl alcohol into the lignin polymers also impacted their interunit linkage patterns, as clearly shown in the difference spectra between the control and line 6 (Fig. 3D). These analyses revealed that resinol III and β-aryl ether I units were notably augmented in both lines compared with the wild-type control, whereas phenylcoumaran II and dibenzodioxocin IV units were relatively depleted, the latter only being observable at lower contour levels; integrals are given on the Fig. 3 plots and in SI Appendix, Table S1. The observed shifts in the lignin linkage patterns reflect the preferential radical coupling modes of SA vs. CA (as discussed below), and thus provide further supporting evidence for the production of S units via radical coupling of SA into the lignins in the transgenic lines.

To determine whether SA synthesized in the transgenic lines is incorporated into lignins via integral copolymerization with the predominant CA to produce G/S lignin copolymers, we performed 2D 1H–13C long-range correlation (HMBC) NMR experiments.

**Table 1. Overview of transgenic lines generated in this study**

| Gene(s)       | No. of lines | No of. lines ¹ | S/G ratio (from pyrolysis-GC/MS)² |
|---------------|--------------|----------------|----------------------------------|
| FSH           | 27           | 11 (41%)       | ≤0.03                            |
| FSH/COMT      | 52           | 27 (52%)       | ≤0.06                            |
| FSH/COMT/SAD  | 26           | 7 (27%)        | ≤0.09                            |

¹ Number of transgenic lines generated.
² The raw data are given in SI Appendix, Table S2.

We used the cell wall dissolution/acyetylation method (32, 33) to prepare acetylated ELs that were completely soluble in deuterchloroform and displayed better relaxation characteristics. HSQC spectra (SI Appendix, Fig. S6) confirmed similar distributions of lignin aromatic and side-chain signals as observed in the spectra of unacetylated EL samples (Fig. 3). HMBC spectra diagnostically revealed expected long-range correlations between the major lignin side-chains and S aromatic rings in the transgenics, as shown, for example, with Line 6 in Fig. 4. Both S and G β-ethers I are evidenced. The correlations between phenylcoumaran II side-chains and S aromatic rings, in particular, provide compelling evidence for direct connections between S and G units, as this structure only derives from cross-coupling of SA with a G phenolic end-unit. Finally, as noted in most dicots, the resinol units III that were obviously solely G in the control were almost entirely S in the transgenic. The evidence that SA is copolymerized with CA and integrally cross-coupled with G units into the G/S lignin copolymer, much like in hardwoods, is therefore compelling.

**Discussion**

**Genes Contributing to Syringyl Lignin Formation in P. radiata.** The generation of S lignin units in P. radiata callus cultures transformed with FSH (Table 1) provided clear evidence that FSH is sufficient to enable SA production. This result can only be explained by the presence of O-methyltransferases in pine that are capable of methylating 5-hydroxyguaiaiacil pathway intermediates (Fig. 1). Cotransformation with FSH and COMT resulted in lines with up to 2–3 times higher S/G ratios than those transformed with FSH alone, and lower levels of pathway intermediates. This strongly suggests that the endogenous O-methyltransferases are unable to accommodate the flux of 5-hydroxyguaiaiacil precursors, and that the introduced COMT contributes to the biosynthesis of S lignin (Fig. 1). Quantitative RT-PCR experiments showed good levels of expression for FSH and COMT in transgenic lines with high S lignin levels (SI Appendix, Fig. S3). High levels of SOH-VG were seen in...
pyrolysis products from the F5H-only transformation experiment. The ability of COMT to prevent build-up of pathway intermediates in pine TEs is important from a biotechnological perspective as 5-hydroxyconiferyl alcohol incorporation into lignins would result in benzodioxane-unit production in lignin (34) that has been shown to have a negative impact on pulping efficiency (35).

SAD, a dehydrogenase conjectured to be involved in the biosynthesis of S units in aspen lignin, is capable of converting sinapaldehyde to SA in vitro (25). Sinapaldehyde is, however, a relatively poor substrate for pine CAD (36), providing the impetus for including SAD in this study. Cotransformation experiments with F5H, COMT, and SAD focused on investigating whether SAD can further improve S levels in pine. SAD expression levels in lines 6 and 19 were extremely low (SI Appendix, Table S2). The fact that screening for high S/G ratios did not select for high levels of SAD expression (contrary to that for F5H and COMT) suggested that this gene did not play a role in promoting SA biosynthesis in pine. In addition, a Student’s t test (SI Appendix, Table S2) showed a statistically significant difference in the S/G ratios ($P < 0.05$) between the F5H lines and the F5H/COMT and F5H/COMT/SAD lines, but no significant difference between the F5H/COMT and F5H/COMT/SAD, suggesting that SAD does not significantly contribute to S-lignin creation in this system. Nevertheless, the two lines, Line 6 and Line 19, with the highest S/G were F5H/COMT/ SAD lines, i.e., with all three constructs. From these results, it is difficult to draw conclusions about the importance of SAD in pine, even though recent evidence shows that the functional ortholog for SAD in N. tabacum is unlikely to be a lignin-related gene and that its $V_{\text{max}}/K_{\text{m}}$ for sinapaldehyde is very low compared with CAD (37).

**Appearance of Syringyl Lignin in Pine TEs.** Incorporation of SA into pine lignin, resulting in production of new S units in the lignins, in the transgenic lines was clearly demonstrated by both pyrolysis-GC/MS and 2D-NMR. Pyrolysis-GC/MS revealed approximately twofold higher S/G ratios (0.15–0.18) compared with those determined by 2D-NMR (0.06–0.09). Such differences are not surprising as NMR measures the S/G of the entire lignin polymer whereas pyrolysis-GC/MS preferentially cleaves and analyzes monomers released from noncondensed interunit linkages in the lignin polymer.

![Fig. 3. Partial short-range 2D $^{1}H$-$^{13}C$ correlation (HSQC) spectra of enzyme lignins (ELs) from transgenic lines F5H/COMT/SAD Line 6 (A) and Line 19 (B), and wild-type control (C), and differential HSQC spectra for F5H/COMT/SAD Line 6 versus wild-type control (D). Volume integrals are given for S and G aromatic units and major lignin substructures that are color-coded to match their signal assignments in the spectra. In the differential spectrum, orange peaks are elevated and the blue peaks are diminished in Line 6 compared with the wild-type control (as also indicated by the up and down arrows).](image-url)
transgenic lines, the expression levels in pine TE cultures do

\( \beta \) regulate the overall lignin structure as revealed by

relation spectra of acetylated enzyme lignins (ELs) from transgenic

Polymer chains in softwood and dicot lignins start from dimers

production of S lignin in the transgenics even though the relative S levels were quite

Factors Limiting Syringyl-Lignin Formation in Pine TEs. Although we

High FSH and COMT expression levels in pine TE cultures do not necessarily equate to high levels of enzymatic activity. FSH for example depends on other protein factors (e.g., P450 reductase), and cofactors such as S-adenosylmethionine (SAM) are required for COMT activity. It is currently unknown how efficiently pine P450 reductases can support FSH activity or whether SAM levels are more limiting in pine compared with hardwoods. FSH activity levels may therefore have been limiting here. It is also possible for the transport of SA to the apoplast to be inefficient in pine. Recent experimental data suggest that ABC transporters involved in monolignol transport can be monolignol-specific (45), which makes it possible that angiosperms have transporters specific for SA (46). The introduction of a SA-specific ABC transporter might further promote S lignin formation in pine. Peroxidases specific for the one-electron oxidation of SA have been identified in angiosperms (26, 47), but are unlikely to exist in conifers. The absence of such peroxidases in pine could compromise the incorporation of SA into the lignin polymer. Finally, suppression studies in *Medicago sativa* indicated
that biosynthesis of SA might involve methyltransferases other than CCoxMT (48). Metabolite profiling in future real S-lignin-generating pine transgenic lines may help in delineating the most important bottlenecks (49).

Conclusions
We have provided proof of the principle that softwood systems can be augmented with the genes/enzymes necessary to biothesize the monolignol sinapyl alcohol (SA), thereby producing syringyl-guaiacyl (S/G) lignins. The potential to develop commercially important softwoods that contain more readily extractable S/G lignins for chemical pulping and in the lignocellulosics-to-biofuels enterprises could be groundbreaking, especially if alteration of the lignin retains the desirable long-fiber characteristics so valuable in softwood pulps.

Materials and Methods
Detailed information on the generation of recombinant constructs, tissue culture procedures, transformation protocols for tracheary element cultures, as well as monitoring, screening and chemical analysis of transgenic lines using quantitative RT-PCR, Pyrolysis-GC/MS, and 2D-NMR are provided in the SI Appendix, Materials and Methods and referenced in Results.

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