Suppression of 4-Coumarate-CoA Ligase in the Coniferous Gymnosperm Pinus radiata

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Severe suppression of 4-coumarate-coenzyme A ligase (4CL) in the coniferous gymnosperm Pinus radiata substantially affected plant phenotype and resulted in dwarfed plants with a “bonsai tree-like” appearance. Microscopic analyses of stem sections from 2-year-old plants revealed substantial morphological changes in both wood and bark tissues. This included the formation of weakly lignified tracheids that displayed signs of collapse and the development of circumferential bands of axial parenchyma. Acetyl bromide-soluble lignin assays and proton nuclear magnetic resonance studies revealed lignin reductions of 36% to 50% in the most severely affected transgenic plants. Two-dimensional nuclear magnetic resonance and pyrolysis-gas chromatography-mass spectrometry studies indicated that lignin reductions were mainly due to depletion of guaiacyl but not 5-hydroxyphenyl lignin. 4CL silencing also caused modifications in the lignin interunit linkage distribution, including elevated β-aryl ether (β-O-4 unit) and spirodienone (β-1) levels, accompanied by lower phenylcoumaran (β-5), resinol (β-8), and dibenzodioxocin (5-5/β-O-4) levels. A sharp depletion in the level of saturated (dihydroconiferyl alcohol) end groups was also observed. Severe suppression of 4CL also affected carbohydrate metabolism. Most obvious was an up to approximately 2-fold increase in galactose content in wood from transgenic plants due to increased compression wood formation. The molecular, anatomical, and analytical data verified that the isolated 4CL clone is associated with lignin biosynthesis and illustrated that 4CL silencing leads to complex, often surprising, physiological and morphological changes in P. radiata.

Lignin is a heterogeneous cell wall polymer derived primarily from hydroxycinnamyl alcohols via combinatorial radical coupling reactions (Ralph et al., 2004). Typically, it makes up 20% to 30% of the cell wall material in woody species and therefore constitutes a significant proportion of plant biomass. Deposition of lignin is of significance for vascular plants, as it reinforces plant cell walls, facilitates water transport, provides compressive strength to conducting tissues, and acts as a mechanical barrier to pathogens (for review, see Boudet, 2007).

A substantial amount of scientific data have been produced in recent years that describe the impact that lignin manipulations can have on plant performance in woody angiosperms (for review, see Boerjan et al., 2003; Halpin, 2004; Chiang, 2006; Higuchi, 2006; Boudet, 2007; Rastogi and Dwivedi, 2008). In recent years, poplar (Populus spp.) has emerged as a model angiosperm tree species for the investigation of wood-related topics, including lignification. For gymnosperms, no such model tree has been identified, and the impact of lignin perturbations on plant performance is still largely unexplored, despite the significant ecological and economic importance of gymnosperm species such as pine (Pinus spp.) trees. Although prior reports describing the metabolic effects of lignin modifications on Pinus radiata tracheary elements have been published (Möller et al., 2005b; Wagner et al., 2007), the first report on lignin modifications in whole conifer plants was published very recently (Wadenbäck et al., 2008).

Coniferous gymnosperms such as pines differ significantly at anatomical, physiological, and biochemical levels from arborescent angiosperms such as poplar. Wood anatomy differs greatly between these groups of tree species (Fig. 1). Pine wood appears less complex and lacks vessel elements, the specialized water-conducting cells found in angiosperm wood. Water conduction and structural support in pine are accomplished via tracheids, which make up the largest component of the wood structure. In addition, lignin composition in pine trees is different from that in angiosperms and other vascular species, including Selaginella, in that it does not contain syringyl units...
Lignin content and composition in pine are also substantially modified during physiological processes such as compression wood formation (Timell, 1982; Nanayakkara et al., 2005). This type of wood is associated with gravitropism in pine (Timell, 1982) and is not formed in arborescent angiosperm species, which instead create tension wood in response to gravitropic stimuli (Hejnowicz, 1997). These profound differences between tree species such as poplar and pine make it scientifically attractive to explore how lignin manipulations affect plant performance and wood formation in coniferous gymnosperms.

4-Coumarate-coenzyme A ligase (4CL) is an enzyme that functions early in the general phenylpropanoid pathway by producing the monolignol precursor p-coumaroyl-CoA (Fig. 2). This metabolite is also a precursor for the production of secondary plant metabolites such as stilbenes and flavonoids (for review, see Boudet, 2007). These multiple functions might explain why 4CL is encoded by a gene family in both angiosperm and gymnosperm species (Lee and Douglas, 1996; Allina et al., 1998; Hu et al., 1998; Harding et al., 2002; Kumar and Ellis, 2003; Costa et al., 2005; Friedmann et al., 2007; Koutaniemi et al., 2007).

4CL silencing in angiosperm species such as tobacco (Nicotiana tabacum), Arabidopsis (Arabidopsis thaliana), and Populus tremuloides caused lignin reductions in the range of 25% to 45% (Kajita et al., 1996, 1997; Lee et al., 1997; Hu et al., 1999; Li et al., 2003). However, the impacts of these manipulations on lignin composition varied. 4CL silencing in tobacco preferentially depleted syringyl (S) lignin units, which are prominent in wood fibers (Kajita et al., 1997). 4CL silencing in Arabidopsis depleted only guaiacyl (G) lignin units, which are enriched in vessel elements (Lee et al., 1997), whereas silencing of 4CL in P. tremuloides had no impact on the S-G ratio (Hu et al., 1999). An increase in the S-G ratio in P. tremuloides was only recorded when 4CL silencing was combined with the over-expression of coniferaldehyde 5-hydroxylase, previously known as ferulate 5-hydroxylase (Li et al., 2003). These differences in lignin composition could be the consequence of silencing 4CL isoforms with different substrate preferences, or they could reflect the inadequacies or limitations of analytical procedures used for lignin analysis (for review, see Halpin, 2004).

In pine species such as Pinus taeda, 4CL expression is stimulated during compression wood formation (Zhang and Chiang, 1997), which implies that elevated 4CL activity is required for increased lignin production. Compression wood in pine has a higher lignin content than normal wood and contains substantial amounts of p-hydroxyphenyl (H) lignin units (Timell, 1982; Nanayakkara et al., 2005). 4CL expression in a P. taeda tissue culture system capable of producing monolignols and extracellular lignin is stimulated by supplying monolignol precursors such as L-Phe.
Expression of 4CL in P. taeda is also subject to feedback inhibition, and its activity is down-regulated by products of the flavonoid and lignin pathways (Voo et al., 1995). In our ongoing efforts to better understand the physiological role of lignin in coniferous gymnosperms, we investigated how 4CL silencing affects plant phenotype, wood anatomy, and chemical wood composition in the conifer species P. radiata.

RESULTS

Clone Isolation and Generation of Transgenic Material

A 1,917-bp fragment of a P. radiata 4CL clone (GenBank accession no. EU616501) containing the entire open reading frame was isolated from a xylem-derived cDNA library using the PCR-based approach described in “Materials and Methods.” The deduced amino acid sequence of the isolated clone was 99.4% identical to its putative P. taeda ortholog (GenBank accession no. PTU12013; Voo et al., 1995; Supplemental Fig. S1) and 91.8% identical to the lignin-related Picea abies 4CL clone Pae4CL3 (EMBL accession no. AM170561; Koutaniemi et al., 2007). Quantitative reverse transcription (RT)-PCR experiments revealed that the expression of the isolated P. radiata 4CL clone substantially increased in concert with other lignin-related genes during tracheary element development (data not shown), which is consistent with a coordinated transcriptional regulation of lignin-related genes in P. radiata (Wagner et al., 2007).

The promoter of the lignin-related P. radiata cinnamyl alcohol dehydrogenase gene was used in the 4CL RNA interference (RNAi) construct (Supplemental Fig. S1) based on its preferential expression in developing xylem (Wagner and Walter, 2004). This was in keeping with the intention of limiting phenotypic alterations to wood-forming tissue. Transformation of embryogenic P. radiata tissue with the 4CL RNAi construct resulted in the generation of 16 transgenic lines. Unfortunately, an unusually high percentage (25%) of the generated transgenic lines died during the late stages of embryo maturation. Twelve transgenic lines were regenerated into plants and grown for a period of 2 years under contained, controlled conditions in a greenhouse prior to analysis.

Figure 2. Biosynthesis of lignin-related phenylpropanoids in pine starting from p-coumarate. HCT, p-Hydroxycinnamoyl-CoA shikimate hydroxycinnamoyltransferase; C3H, p-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; PPDBR, phenylpropanal double-bond reductase; ?, unknown.
played an essentially wild-type phenotype, and the other two transgenic lines were dwarfed. Three transgenic lines (AW42-13, AW42-14, and AW42-17) displayed reduced lignin levels in pyrolysis-gas chromatography-mass spectrometry (GC-MS) experiments and quantitative acetyl bromide lignin assays and therefore were selected for further studies.

**Molecular and Anatomical Characterization of 4CL Transgenic Lines**

Quantitative RT-PCR experiments revealed that transgenic lines AW42-13, AW42-14, and AW42-17 contained substantially reduced 4CL steady-state mRNA pools in developing xylem, which represented 5%, 8%, and 21% of the 4CL message, respectively, of wild-type plants grown under the same conditions.

The two lines that showed substantial phenotypic abnormalities, AW42-13 and AW42-14, were characterized by dwarfing and the absence of a straight, dominant leader that is otherwise typical for wild-type *P. radiata* plants (Fig. 1C). Line AW42-17 displayed a wild-type phenotype (data not shown). Branches on AW42-13 and AW42-14 appeared more variable in form compared with those of wild-type plants, which added to the bonsai tree-like appearance of these transgenic lines (Fig. 1C). Wood from stem and branch material of AW42-13 and AW42-14 plants, but not from AW42-17 plants, was darker in color compared with wood from control trees, which indicated substantial changes in wood anatomy and chemistry (Fig. 1D).

Microscopic investigations revealed substantial changes in the anatomy of the stems formed in AW42-13 and AW42-14 (Figs. 3–5). Stem sections from AW42-17 were indistinguishable from those of wild-type plants (data not shown). Stem sections from AW42-13 and AW42-14 had a higher proportion of bark relative to wood (40%–60% versus 24% in the control), which was most evident in the most severely suppressed line, AW42-13 (Fig. 3C). Wood from AW42-13 and AW42-14 also contained circumferential bands of axial parenchyma (Figs. 3B and 4, G and H). These axial parenchyma cells were mostly un lignified, but weakly lignified cells occasionally occurred within this tissue (Fig. 4, G and H). Axial parenchyma tissue was similar in appearance to the cells surrounding resin canals in normal *P. radiata* wood (Bamber, 1972). However, the virtual absence of resin canals in those tissues indicated that this tissue differs from the traumatic bands of resin canals that are formed as a response to injury in conifers (Nagy et al., 2000; Martin et al., 2002). Stem segments of AW42-13 and AW42-14 also contained elevated levels of compounds, potentially flavonoids or tannin-like compounds, in both wood and bark tissues, which formed dark-colored complexes with FeCl₃ (Fig. 3B).

Confocal fluorescence microscopy was used to visualize the degree of lignification of wood tissue using the lignin stains basic fuchsin and berberine sulfate (Fig. 4). Both staining techniques delivered comparable results. Normal and compression wood tissues in line AW42-17 were indistinguishable from control material (data not shown). However, transgenic lines AW42-13 and AW42-14 showed regions of reduced levels of lignification. The compound middle lamella and the outer part of the secondary wall were lignified to at least a moderate extent, but the inner secondary wall was either un lignified or weakly lignified (Fig. 4). Affected tracheids were in clusters or radial files interspersed with apparently normal tracheids and tracheids with varying levels of decreased lignification (Fig. 4B). Tracheids adjacent to resin canals and wood rays exhibited increased fluorescence when stained with basic fuchsin or berberine sulfate (Fig. 4, B, C, E, and F), and similar variability in the staining pattern was also observed with Wiesner reagent (Fig. 5). Tracheids severely affected by lignin depletion showed signs of collapse (Figs. 4I and 6A) and a lack of cell adhesion, which was most obvious in transmission electron micrographs (Fig. 6A). UV micrographs indicated that in these tracheids, lignification was severely reduced in all regions of the cell wall, with only the middle lamella and S1 layers showing moderate levels of lignification (Fig. 7).

**Biochemical Changes in Woody Tissue of 4CL Transgenic Lines**

Extracted wood samples from nontransgenic controls and transgenic lines AW42-13, AW42-14, and AW42-17 were analyzed using pyrolysis-GC-MS to generate a chemical fingerprint of their cell wall composition. The pyrograms of controls and transgenic lines displayed a number of characteristic differences. These are most easily visualized by comparing control material with the most severely suppressed line, AW42-13 (Fig. 8). Most obvious were the decreased signals for vanillin, coniferaldehyde, conifer alcohol, and dihydroconiferyl alcohol in transgenic material (Fig. 8; Table I). Many of the signals reduced in transgenic lines, including those mentioned above, represented derivatives of G-type lignin. Pyrolysis products diagnostic for H-type lignin were not reduced in transgenic lines. The H-G ratio in transgenic lines with severe phenotypes, such as AW42-13 and AW42-14, was consequently up to 3-fold higher than that in wild-type controls. Transgenic line AW42-17 displayed a virtually unchanged H-G ratio, most likely due to its weak phenotype.

Quantitative acetyl bromide-soluble lignin (ABSL) measurements were used to verify the trends in lignin content observed in pyrolysis-GC-MS experiments. These experiments revealed that lignin content in AW42-13, AW42-14, and AW42-17 was decreased on average by 36%, 28%, and 8% relative to control plants (Table II). ABSL content in the 10 control plants analyzed in this study varied between 26.0% and 30.9% (w/w). Lignin content in the control plants was at least
4 SDs higher than that in the dwarfed transgenic lines AW42-13 and AW42-14 (Table II).

Parallel ABL and Klason measurements were performed in a subset of five control plants, which produced virtually identical results. The average lignin content was 28.0% ± 1.6% (w/w) for Klason lignin and 28.1% ± 1.6% (w/w) for ABL. The maximum difference in lignin content for a given plant was 0.8% (w/w). These results demonstrate that the ABL data generated in this study can be compared with other published data, which are based on Klason lignin.

4CL silencing in P. radiata affected not only lignin content and composition but also the polysaccharide composition in wood of AW42-13 and AW42-14. Most obvious was the increase in Gal released from ground wood, which most likely originated from galactan, since the Man content in those transgenic lines was inconsistent with an increase in galactoglucomannan, the second major source of Gal in pine wood (Table II). Slight alterations in Ara and Xyl contents were also observed.

Compositional and Structural Changes in Lignin of 4CL Transgenic Lines

One-dimensional NMR spectroscopy and two-dimensional $^{13}$C-$^1$H correlation (HSQC) NMR studies with the most severely affected transgenic line, AW42-13, confirmed the compositional changes observed in the pyrolysis-GC-MS experiments. Evident in all spectra, but most easily illustrated in the one-dimensional proton NMR spectra of the acetylated cell wall material, was the approximately 2-fold reduction in lignin content in AW42-13 (Supplemental Fig. S2). More accurate quantification was not possible, as proton resonances from certain H units were obscured by the residual nondeuterated NMR solvent (CHCl$_3$). Similar trends were also observed in two-dimensional spectra, which were adjusted to match cellulose levels (Supplemental Figs. S4–S6). Spectra derived from AW42-13 displayed significant reductions in lignin contours relative to those from cellulose (Supplemental Figs. S4–S6).

The lignin monomer compositions were determined via volume integration of contours in the HSQC spectra and were measured as described previously (Wagner et al., 2007), except that an adiabatic pulse variant of the HSQC experiment was used to improve quantification (see “Materials and Methods”). The H-G ratio was greater than 4-fold higher in AW42-13 compared with the control (Table III) and therefore similar to H-G ratios found in pine compression wood (Nanayakkara et al., 2005). This relative increase in H lignin in AW42-13 was most easily visualized when the guaiacyl-2 C/H correlations were set to be approximately equivalent (Supplemental Fig. S3). This trend could also be observed in Supplementary Figure S6, which was adjusted to match cellulose levels. Here, AW42-13 displayed substantially depleted G-lignin but essentially unchanged H-lignin levels. As anticipated from the increased H contribution to the lignin, the methoxyl-aromatic ratio, measured by integrating the methoxyl contour versus the G2 and H2/6 aromatic contours, was approximately 6% lower in AW42-13 compared with the wild-type control.

Figure 3. Images of P. radiata stem segments from a wild-type control (left images) and 4CL-RNAi lines AW42-14 (middle images) and AW42-13 (right images). A, Unstained images of cut stems. B, Stem sections stained with FeCl$_3$ showing tannins as black-stained areas. C, Stem sections stained with phloroglucinol HCl showing lignified tissue stained red. Bar = 1 cm.
Lignin structural changes are best described by comparing interunit and end unit profiles in HSQC spectra. Table III lists relative quantification data from integrating the correlations from the various lignin units, as described previously (Wagner et al., 2007). The units measured are shown in Figure 9, which displays partial HSQC spectra (side-chain region only) of cellulolytic enzyme lignins from AW42-13 and a control sample. The yield of cellulolytic enzyme lignins in AW42-13 was 19% (w/w), compared with 28% (w/w) for the control. Note that the contour levels used to display the two spectra were chosen to highlight the lignin structural similarities and differences and therefore do not reflect the fact that the transgenic line had a lower total lignin content. The β-ether Aα contour levels were set to be approximately equivalent. β-Ether and spirodienone units were elevated in AW42-13 was 19% (w/w), compared with 28% (w/w) for the control. Note that the contour levels used to display the two spectra were chosen to highlight the lignin structural similarities and differences and therefore do not reflect the fact that the transgenic line had a lower total lignin content. The β-ether Aα contour levels were set to be approximately equivalent. β-Ether and spirodienone units were elevated in AW42-13 at the expense of phenylcoumaran, resinol, and dibenzodioxocin units (Fig. 9; Table III). Also notable was the substantial depletion in the level of reduced dihydroconiferyl alcohol end groups, as determined by the Hα/Cα and Hβ/Cβ correlations at 2.60/31.7 and 1.90/30.3 ppm (data not shown; Ralph et al., 1999).

DISCUSSION

Effect of 4CL Silencing on Plant Anatomy and Physiology

NMR data indicate that severe (95%) suppression of 4CL can lead to an approximately 50% reduction in lignin content in pine plants (Supplemental Fig. S2). This demonstrates that 4CL has a key role in the biosynthesis of monolignols in softwood species such as *P. radiata*. The same is true for hardwood species such as *P. tremuloides*, in which similar reductions in lignin content were recorded in 4CL-silencing experiments (Hu et al., 1999). However, transgenic aspen (*Populus* spp.) retained a wild-type-like appearance despite these substantial lignin depletions (Hu et al., 1999; Li et al., 2003), which was clearly not the case in this study. Only moderate lignin reductions of approximately 8% could be tolerated without apparent effect on plant phenotype in pine. The more substantial lignin reductions measured in AW42-13 and AW42-14 severely affected plant phenotype, including bark and wood anatomy (Figs. 1 and 3–7).

The collapse of water-conducting elements when lignin production is experimentally suppressed appears to be a significant factor contributing to growth.
retardation in tree species (Leplé et al., 2007; this study). Water-conducting tracheids are the primary cell type in pine wood (Fig. 1A). Consequently, lignin manipulations targeting wood formation in pine will almost inevitably affect the lignification of tracheids, which has the potential to compromise their anatomical integrity and, thereby, their function (Figs. 4I and 6A). In contrast, substantial lignin reductions were achieved in aspen without causing the collapse of water-conducting vessel elements (Hu et al., 1999, Li et al., 2003). It thus appears that phenotype formation in hardwood species might depend to some extent on whether lignin manipulations primarily affect conducting or structural elements in developing xylem.

Such discrimination is impossible in softwood species such as pine, since tracheids combine both water-conducting and structural functions, which may effectively restrict the potential for lignin reductions in pine species.

Physiological as well as anatomical differences between hardwoods and softwoods are also likely to contribute to the drastically different 4CL-silencing phenotypes observed in aspen and pine. Unlike hardwoods, lignification plays an important role in gravitropism in conifers. Compression wood, which contains a high lignin content, forms on the lower side of branch and stem material in conifers in response to gravitropic stimuli (Timell, 1982). In contrast, the corresponding gravitropic response in hardwoods is the deposition of tension wood, which is rich in cellulose, not lignin (Hejnowicz, 1997). The 4CL-silencing phenotypes we observe thus appear to be indicative of the more crucial role lignin biosynthesis plays in growth and development in softwoods relative to hardwoods.

The elevated Gal levels in severely 4CL-silenced transgenic pine plants (Table II) might also be linked to the gravitropic response. High Gal levels are, as men-

**Figure 5.** Images of *P. radiata* stem sections from a wild-type control (A) and 4CL-RNAi line AW42-14 (B and C) treated with Wiesner reagent showing uniform lignification in the wild type and variable lignification in the transgenic material. Field of view: 500 × 730 μm.

**Figure 6.** Transmission electron micrographs of *P. radiata* stem sections from 4CL-RNAi line AW42-13 stained with potassium permanganate showing collapsed tracheids with poorly lignified cell walls (A) and a wild-type control showing normal tracheids (B). Bars = 4 μm (A) and 10 μm (B).
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**Figure 7.** UV micrographs of stem sections from line AW42-13 showing partially collapsed tracheids (top image) and a wild-type control (bottom image) scanned at a wavelength of 280 nm at a resolution of 0.25 μm². The transgenic line displayed substantial reductions in UV absorption in all layers of the cell wall compared with the control.

mentioned earlier, most likely due to increased galactan levels in the transgenic plants. Increased galactan content was discernible in AW42-13 in two-dimensional NMR experiments but could not be quantified, as the most distinct correlation for galactan partially overlapped with that from cellulose and xylan. Galactan is closely associated with compression wood formation and, therefore, the gravitropic response in conifers (Jiang and Timell, 1972; Nanayakkara et al., 2005). 4CL-silencing experiments in tracheary element-forming *P. radiata* callus cultures did not lead to a similar increase in Gal content, despite reductions in lignin content of up to 60% (A. Wagner, K. Torr, L. Phillips, and H. Flint, unpublished data). This demonstrates that increased galactan production is not an obligatory consequence of 4CL silencing in *P. radiata* but rather a possible gravitropic response in structurally weakened plants due to lignin reduction. The physiological role of galactan in pine compression wood is currently unresolved. However, the bonsai tree-like phenotype of transgenic lines such as AW42-13 (Fig. 1C) might suggest that both elevated galactan and lignin levels are integral to the gravitropic response in pine.

Wood and bark tissues of AW42-13 and AW42-14 contained elevated levels of metabolites or polymers, likely flavonoids or derivatives of flavonoids such as condensed tannins, that form dark-colored complexes with FeCl₃ (Fig. 3). In particular, tannins containing pyrogallol units readily form complexes with FeCl₃ (Sungur and Uzar, 2008), and condensed tannins containing pyrogallol groups have been identified in pine previously (Ku and Mun, 2007). The presence of increased levels of flavonoids or flavonoid derivatives in transgenic pine plants would not be surprising, since elevated flavonoid production is quite common when silencing genes early in the monolignol pathway (Chen et al., 2006; Besseau et al., 2007). Such a perturbation can be the consequence of a redirection of metabolites or indicative of a stress response. The biosynthesis of flavonoids, condensed tannins, and lignin in pine requires 4CL activity. If the dark-colored complexes represent flavonoids or condensed tannins, then this would suggest that 4CL activity is still present in those tissues. 4CL in pine, as in angiosperms, is encoded by multiple genes (Friedmann et al., 2007; Koutaniemi et al., 2007), and 4CL genes participating in different phenylpropanoid-derived pathways have been identified (Kumar and Ellis, 2003). Thus, the production of flavonoids or condensed tannins in axial parenchyma and bark could depend on a member of the 4CL gene family in pine, which is different enough from the targeted lignin-related 4CL gene to not be affected by the RNAi construct.

Severe lignin reductions in pine affected not only the biochemical composition of wood and plant growth but also bark and wood formation itself (Fig. 3C), which was unexpected. The increased bark formation in transgenic plants might be indicative of a redirection of carbon flux from xylem to phloem formation. Similarly, the generation of axial parenchyma in woody tissue (Fig. 4) was surprising, and further studies are required to understand the mechanisms leading to the formation of this tissue.

**Effect of 4CL Silencing on Lignin Composition and Structure**

In addition to significantly reducing lignin levels, 4CL silencing also affected lignin composition in *P. radiata*. A substantial reduction of G units compared with H units was observed in NMR and pyrolysis-GC-MS spectra in affected transgenic plants, which resulted in H-G ratios similar to those of mild pine compression wood (Nanayakkara et al., 2005). It is conceivable that restricting monolignol biosynthesis in *P. radiata* primarily affects G units simply because this type of lignin represents the vast majority (approximately 98%) of lignin in normal pine wood. However, the phenotype of transgenic plants might also have contributed to the observed shift in the H-G ratio. Leaning stems trigger compression wood formation in wild-type plants, which are known to contain high levels of H-type lignin (Timell, 1982; Nanayakkara et al., 2005). 4CL silencing certainly compromised normal compression wood formation in transgenic lines by limiting monolignol supply. However, transgenic lines with a bonsai tree-like appearance might have produced an H-G ratio that is more consistent with compression wood than normal wood, due to their tilted stems.

Changes in lignin composition also cause structural changes in the lignin polymer in hardwood species.
for review, see Ralph et al., 2004), and this study was consistent with earlier findings in that regard (Fig. 9; Table III; Supplemental Figs. S2–S6). Reduction in G-type lignin resulted in lower levels of phenylcoumarans, resinols, and dibenzodioxocins. H units can form such structures but in different distributions, and the correlations may be shifted, especially for dibenzodioxocins (Ralph et al., 2006). These reductions were compensated by an apparent increase in the fraction of \(\beta\)-ethers and spirodienones (from \(\beta\)-O-4 and \(\beta\)-1 coupling). One possible explanation for this change in interunit distribution in AW42-13 might be associated with a reduced monomer supply. Restricting monomer supply results in fewer monomer-monomer reactions (e.g. \(\beta\)-\(\beta\) coupling to generate resinol units) and enhances monomer-polymer cross-coupling in a linear manner, which favors the generation of \(\beta\)-ether units. This fits the Syrjänen and Brunow (2000) model that limiting monomer diffusion enhances cross-coupling and polymer extension.

![Figure 8. Pyrogram (total ion chromatogram) of powdered and extracted P. radiata stem material from 4CL-RNAi line pAW42-13 (A) and a wild-type control (B). Signals 1 to 35 refer to the following pyrolysis products. 1, (3H)-furan-2-one; 2, dihydro-methylfuranone (isomer); 3, dihydro-methyl-furanone (isomer); 4, 2-hydroxy-1-methyl-1-cyclopentene-3-one; 5, guaiacol; 6, 4-hydroxy-5,6-dihydro-2H-pyran-2-one; 7, 4-methyl-guaiacol; 8, phenol; 9, 4-ethyl-guaiacol; 10, dimethyl-phenol; 11, eugenol; 12, 4-vinyl-guaiacol; 13, 4-hydroxy-3-methyl-3H-furanone; 14, unknown; 15, cis-isoeugenol; 16, trans-isoeugenol; 17, 5-hydroxymethyl-2-furaldehyde; 18, vanillin; 19, 1,5-anhydro-arabinofuranose; 20, homovanillin; 21, acetoguaiacone; 22, 3-methyl-catechol; 23, catechol; 24, unknown; 25, 4-methyl-catechol; 26, unknown; 27, 1,5-anhydro-\(\beta\)-n-xylofuranose; 28, dihydroconiferyl alcohol; 29, 1,6-anhydro-a-D-galactopyranose; 30, coniferaldehyde; 31, 1,6-anhydro-\(\beta\)-o-mannopyranose; 32, unknown; 33, trans-coniferyl alcohol; 34, unknown; 35, 1,6-anhydro-\(\beta\)-o-glucopyranose. A series of signals diagnostic for guaiacyl-lignin (16, 18, 28, 30, and 33) were substantially reduced in pAW42-13 compared with the wild-type control.]

Table 1. Signal surface area for selected pyrolysis products from wood samples of a wild-type control and transgenic lines AW42-13, AW42-14, and AW42-17

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vanillin, (m/z) 151</th>
<th>Coniferaldehyde, (m/z) 178</th>
<th>Coniferyl Alcohol, (m/z) 180</th>
<th>Dihydroconiferyl Alcohol, (m/z) 182</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>124,095 ± 16,815</td>
<td>80,492 ± 3,221</td>
<td>77,034 ± 13,245</td>
<td>20,996 ± 907</td>
</tr>
<tr>
<td>AW42-13</td>
<td>59,872 ± 19,773</td>
<td>33,668 ± 8,989</td>
<td>21,769 ± 5,559</td>
<td>6,130 ± 2,083</td>
</tr>
<tr>
<td>AW42-14</td>
<td>70,685 ± 18,741</td>
<td>36,926 ± 2,273</td>
<td>31,018 ± 1,909</td>
<td>10,339 ± 636</td>
</tr>
<tr>
<td>AW42-17</td>
<td>105,888 ± 11,993</td>
<td>71,687 ± 8,747</td>
<td>58,164 ± 4,372</td>
<td>15,891 ± 3,803</td>
</tr>
</tbody>
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Also notable, particularly from the reduced Ca/Ha and Cβ/Hβ correlations (data not shown), is the substantial reduction in the level of reduced (dihydroconiferyl alcohol) end groups, which was also observed in pyrolysis-GC-MS experiments (Fig. 8). Such groups are derived from dihydroconiferyl alcohol monomers that are incorporated into lignin (Ralph et al., 1997, 1999; Sederoff et al., 1999). Dihydroconiferyl alcohol itself is likely to be a derivative of coniferaldehyde in pine (Savidge and Forster, 2001; Kasahara et al., 2006). 4CL silencing in P. radiata preferentially affected G-type lignin (Supplemental Figs. S4 and S6) and thus probably the biosynthesis of coniferaldehyde. Limiting coniferaldehyde biosynthesis in pine also restricted the biosynthesis of both coniferyl alcohol and dihydroconiferyl alcohol (Fig. 2).

**Phenotypic Nonuniformity in Transgenic Plants**

Lack of phenotypic uniformity in gene-silencing experiments in plants is a fairly common phenomenon. This phenotypic inconsistency, also sometimes referred to as “patchiness,” has been reported for hardwood species previously (Baucher et al., 1996; Tsai et al., 1998; Meyermans et al., 2000; Pilate et al., 2002; Leplé et al., 2007). This study revealed that patchiness in gene-silencing experiments can also occur in softwood species, based on the observation that lignin reductions in tracheids were far from uniform in transgenic plants (Figs. 4B and 5B). The reason for this lack of phenotypic uniformity is currently unclear, but it could be associated with the nature of the RNAi process. RNAi in plants is based on both cell-autonomous and non-cell-autonomous processes (Shimamura et al., 2007), which might allow for differential responses to occur at the cellular level. RNAi can also be affected by developmental processes in pine (Wagner et al., 2005, 2007). These and other factors, therefore, might affect the phenotype at the cellular level in wood. The observation that phenotypic differences occur at the cellular level in silencing experiments might have implications for biotechnological applications, especially in situations in which phenotypic uniformity is a crucial performance criterion.

Based on the observation that tracheids adjacent to resin canals and wood rays were less severely affected by 4CL silencing than those more distant from those tissues (Fig. 4, B, C, E, and F), some phenotypic variation might be explained by the production of phenylpropanoids in these nonlignifying tissues. Resin canals and wood rays in different pine species produce extractives, some of which are derivatives of ferulic acid (Harborne, 1980). A multifunctional pine O-methyltransferase, which is capable of producing ferulate in vitro (Li et al., 1997), is strongly expressed in wood rays and resin canals in P. radiata (Wagner and Walter, 2004). It is conceivable that phenylpropanoids produced in wood rays and resin canals can be incorporated into the cell wall of tracheids adjacent to those tissues, thereby mitigating the impact of 4CL suppression in those cells. The incorporation of ferulate into lignin of angiosperm species such as tobacco and Populus tremula × Populus alba was recently demonstrated (Dauwe et al., 2007; Leplé et al., 2007; Ralph et al., 2008).

### Table II. ABSSL (% w/w) and neutral sugar content (% w/w) in wood of a wild-type control and transgenic lines AW42-13, AW42-14, and AW42-17

All values represent averages ± SD of at least two independent measurements, except as indicated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ABSSL</th>
<th>Ara</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.8 ± 1.4</td>
<td>2.0 ± 0.1</td>
<td>2.4 ± 0.3</td>
<td>39.0 ± 1.0</td>
<td>8.3 ± 0.3</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>AW42-13</td>
<td>18.5 ± 1.9</td>
<td>2.9 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>40.2 ± 0.6</td>
<td>5.7 ± 0.1</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>AW42-14</td>
<td>20.7 ± 1.7</td>
<td>2.7 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>40.2 ± 0.8</td>
<td>7.8 ± 0.2</td>
<td>10.8 ± 0.4</td>
</tr>
<tr>
<td>AW42-17</td>
<td>26.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>38.6 ± 0.6</td>
<td>8.0 ± 0.1</td>
<td>10.9 ± 0.1</td>
</tr>
</tbody>
</table>

*This value represents the average of 10 independent measurements.

### Table III. NMR-derived p-hydroxyphenyl/guaiacyl (H-G) data and interunit linkage data for lignin from a wild-type control and transgenic line AW42-13

H, p-Hydroxyphenyl; G, guaiacyl; A, β-O-4 (β-aryl ether); B, β-5 (phenylcoumaran); C, β-β (resinol); D, β-O-4/5-5 (dibenzoquinolizoxin); S, β-1 (spiridonione); X5, dihydroconiferyl alcohol end group (see Fig. 9 for structures, except X5, the hydrogenated [double bond-saturated] analog of X1, which is not shown and is not visible in the plotted region of these spectra). Note that H-G values were determined from the solubilized whole cell wall fraction; interunit linkage distribution was determined from polysaccharidase-treated walls (cellulolytic enzyme lignin fraction).

<table>
<thead>
<tr>
<th>Sample</th>
<th>%H</th>
<th>%G</th>
<th>H-G</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
<th>%D</th>
<th>%S</th>
<th>%X5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.4</td>
<td>96.6</td>
<td>0.03</td>
<td>76</td>
<td>15</td>
<td>5.2</td>
<td>3.6</td>
<td>0.1</td>
<td>3.1</td>
</tr>
<tr>
<td>AW42-13</td>
<td>13.5</td>
<td>86.5</td>
<td>0.16</td>
<td>82</td>
<td>12</td>
<td>3.6</td>
<td>2.3</td>
<td>0.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Figure 9. Partial short-range $^{13}$C-$^1$H (HSQC) spectra (side-chain regions) of acetylated cellulolytic enzyme lignins isolated from the wild-type control (A) and 4CL-deficient line AW42-13 (B). 4CL deficiency and the incorporation of relatively higher levels of $p$-coumaryl alcohol into the lignin produces changes in the distribution of interunit linkage types. Volume integrals and semiquantitative data are given in Table III. Interunit type designations A to D, S, and X1 (and X5; not shown here) follow conventions established previously (Boerjan et al., 2003; Ralph et al., 2004, 2006; Wagner et al., 2007).
CONCLUSION

Severe suppression of 4CL in P. radiata plants resulted in some expected phenotypic changes, including a reduction in lignin content and changes in lignin composition, but also in a number of more surprising phenotypic effects. Some of these effects, such as changes in the wood-bark ratio, may be associated with altered metabolic flux of phenylpropanoids. Decreased wood formation may also reflect a certain dependence of xylogenesis in pine on an adequate supply of “building blocks” such as lignin precursors, and increased bark production may be the consequence of restricted xylem formation. Other phenotypes, such as the generation of axial parenchyma in wood and changes in carbohydrate metabolism, likely have explanations originating from other aspects of the physiology of the species. In any case, these pleiotropic phenotypes imply a degree of physiological complexity in pine that is currently not well understood. Parallel testing of lignin-related genes in plants and the pine tracheal element system can help to identify whole plant changes, as opposed to merely cellular phenotypic changes, such as the elevated production of Gal observed in this study. Finally, our results highlight the fact that lignin modifications in pine plants result in metabolic and physiological changes in pine that could not have been predicted from similar experiments in hardwood species. Our findings provide an early indication that lignin biosynthesis might play a more essential role in conifer development and physiology than it does in arborescent angiosperm species. Clearly, many more studies will be required to fully understand this role in gymnosperms.

MATERIALS AND METHODS

Clone Isolation, Construct Design, and Transformation

A 1,917-bp PCR fragment of a P. radiata 4CL cDNA clone was isolated from a xylem-derived cDNA library using primer pair 4CLfwd (5'-CAT- TCAATCTCCACTGAGG-3') and 4CLrev1 (5'-CAAGAGTGAAGGCGTTGACAACT-3'), which were designed using preexisting sequence information from P. taeda 4CL clone PTU12013 (Voo et al., 1995). The amplified PCR fragment of the P. radiata 4CL cDNA clone was cloned into pGEM-T Easy (Promega) and sequenced. A central 1,383-bp EcoRI fragment spanning approximately 85% of the protein-coding region of the 4CL clone was subsequently inserted in the sense and antisense orientations into a derivative of pAH25 (Christensen et al., 1992), which contained the P. radiata cinnamyl alcohol dehydrogenase promoter (Wagner and Walter, 2004) instead of the Zea mays Ubi1 promoter. The resulting plasmid containing the final 4CL RNAi construct was named pAW42 (Supplemental Fig. S1).

Embryogenic P. radiata cultures were cotransformed with pAW42 and pAW16 (Wagner et al., 2007) as described earlier (Walter et al., 1998). The expression of the targeted 4CL clone in developing tracheal elements and differentiating xylem of transgenic and control plants was monitored by quantitative RT-PCR as described previously (Cato et al., 2006) using primers 4CLfwd (5'-TGCGAGATGAAAGCCCTATAA-3') and 4CLrev (5'-GTAAGGGCGTTGACAACTC-3').

Microscopic Analyses

Stem segments of 2-year-old plants including bark, phloem, cambium, and xylem were fixed and stored in formalin acetoalcohol. Transverse sections 60 μm in thickness were prepared using a sledge microtome. Sections for light microscopy were stained with chlorogenic acid HCl (for lignin) or with FeCl₃ (for tannin-like material) and examined using a Leica MZ12.5 stereomicroscope. The proportion of wood to bark in stained stem sections was measured by digital image analysis by comparison of the relative areas. Sections prepared for confocal fluorescence microscopy were stained with aralin (0.0025%, 5 min), basic fuchsin (0.001%, 5 min), or berberine sulfate (0.01%, 5 min) and mounted in glycerol or immersion oil after drying as described earlier (Donaldson and Bond, 2005). For transmission electron microscopy, small blocks of xylem (2 × 2 × 3 mm) were prepared, dehydrated in an acetone series, and embedded in Spurr resin (Spurr, 1969). Transverse sections were prepared using a diamond knife at either 90 or 120 nm thickness, transferred to copper grids, and stained with potassium permanganate (1% in 1% sodium citrate). Sections were examined using a JEOI 6700F field emission scanning electron microscope equipped with a transmission detector at 30 kV. Sections for UV microspectrophotometry were prepared as described earlier (Möller et al., 2005a).

Quantitative Lignin Measurements

ABSL assays were carried out essentially as described by Wagner et al. (2007) with the exception that powdered, freeze-dried stem material was extracted with 4:1 ethanol:water (40 mL g⁻¹) and 2:1 chloroform:methanol (40 mL g⁻¹) according to Hatfield et al. (1999) prior to analysis. Acid-insoluble Klason lignin in extracted (see above) wood samples was determined by the method of Efland (1977). Acid-soluble lignin was determined as described by Dence (1992).

Quantification of Neutral Sugars

The neutral sugar content in stem material from 2-year-old transgenic plants and nontransformed controls was determined as described (Petterson and Schwandt, 1991).

Pyrolysis-GC-MS

Pyrolysis-GC-MS was essentially carried out as described by Möller et al. (2003) with the exception that powdered and freeze-dried stem material was extracted as described by Hatfield et al. (1999) prior to pyrolysis experiments. Pyrolysis products were separated on a Zebron ZB-WAXplus column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Phenomenex), which produces superior signals for polysaccharide- and flavonoid-derived pyrolysis products. Pyrolysis products were identified using mass spectra of lignin and polysaccharide-derived pyrolysis products (Faux et al., 1990, 1991a, 1991b; Ralph and Hatfield, 1991).

NMR Spectroscopy

Preparation of whole cell wall and cellulolytic enzyme lignin samples for NMR was as described previously (Lu and Ralph, 2003; Wagner et al., 2007), as were the NMR methods, with the exception that soft 180 adiabatic pulse variants of the HSQC experiments (Kuiper and Freeman, 2007) were used (Bruker pulse program hsqcquadpsip2). Such experiments are less sensitive to differences in one-bond 13C-1H coupling constants and the response over the entire spectral range is more uniform, suggesting that improved quantification should result.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU616801.

Supplemental Material

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of the Pr4CL coding region with putative orthologs from other conifer species, and schematic diagrams of constructs used in this study.

Supplemental Figure S2. Proton NMR spectra of acetylated whole cell wall preparations from a wild-type control and transgenic line AW42-13.
**LITERATURE CITED**


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