

Antisense Suppression of 4-Coumarate:Coenzyme A Ligase Activity in Arabidopsis Leads to Altered Lignin Subunit Composition

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The phenylpropanoid enzyme 4-coumarate:coenzyme A ligase (4CL) is considered necessary to activate the hydroxycinnamic acids for the biosynthesis of the coniferyl and sinapyl alcohols subsequently polymerized into lignin. To clarify the role played by 4CL in the biosynthesis of the guaiacyl (G) and syringyl (S) units characteristic of angiosperm lignin, we generated 4CL antisense Arabidopsis lines having as low as 8% residual 4CL activity. The plants had decreases in thioglycolic acid-extractable lignin correlating with decreases in 4CL activity. Nitrobenzene oxidation of cell walls from bolting stems revealed a significant decrease in G units in 4CL-suppressed plants; however, levels of S lignin units were unchanged in even the most severely 4CL-suppressed plants. These effects led to a large decrease in the G/S ratio in these plants. Our results suggest that an uncharacterized metabolic route to sinapyl alcohol, which is independent of 4CL, may exist in Arabidopsis. They also demonstrate that repression of 4CL activity may provide an avenue to manipulate angiosperm lignin subunit composition in a predictable manner.

INTRODUCTION

Lignin is a complex phenolic polymer based on cinnamyl alcohol subunits derived from phenylpropanoid metabolism and is a major plant natural product. Ubiquitous in vascular plants, lignin plays important roles in structural support, water transport, and defense (see reviews in Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995; Campbell and Sederoff, 1996; Douglas, 1996). Lignin deposition is restricted to a limited number of cell types, such as tracheary elements in the xylem and sclerenchyma, and is usually deposited in secondarily thickened walls of these cells. In woody plants, where secondary xylem forms the bulk of the plant body, lignin biosynthesis requires a large biosynthetic commitment, and certain organs of herbaceous plants also become heavily lignified. Thus, the regulation of lignin biosynthesis is critical to plant growth and development.

Depending on plant species and cell type, the lignin polymer can be composed of differing amounts of *p*-hydroxyphenyl, guaiacyl, and syringyl monomeric subunits. In dicotyledonous angiosperms, guaiacyl (G) and syringyl (S) subunits predominate; in conifers, lignin consists almost exclusively of G units. As shown in Figure 1, these units are derived from cin-

namyl alcohols (monolignols), which in turn are derived from hydroxylated and methoxylated derivatives of cinnamic acid. Monolignol transport to the cell wall is followed by free radical-mediated polymerization, resulting in deposition of a large, heavily cross-linked lignin polymer of undefined size.

Most of the enzymes required for the synthesis of lignin precursors have been characterized, and many of the corresponding genes have been cloned (reviewed in Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995; Boudet and Grima-Pettanati, 1996; Campbell and Sederoff, 1996; Douglas, 1996; see Figure 1). Key steps include hydroxylation reactions catalyzed by cinnamate-4-hydroxylase (C4H) and ferulate-5-hydroxylase (F5H) and methylation reactions catalyzed by the bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) (Figure 1A). Reduction of ferulic acid and sinapic acid to their corresponding alcohols occurs in three steps: formation of activated thioesters by the action of 4-coumarate:coenzyme A ligase (4CL), and reduction to the aldehydes and alcohols by the actions of cinnamyl-CoA reductase and cinnamyl alcohol dehydrogenase (CAD), respectively (Figure 1C). Alternatively, methylation can occur at the level of the CoA esters rather than that of the free acids (Figure 1B) by the action of caffeoyl-CoA *O*-methyltransferase (CCoOMT) acting on caffeoyl-CoA and possibly 5-hydroxyferuloyl-CoA (Ye et al., 1994), or even later by the actions of *O*-methyltransferases on hydroxycinnamyl aldehydes or alcohols (Figure 1C; Whetten and Sederoff et al., 1995).

Lignin monomer composition varies according to cell type

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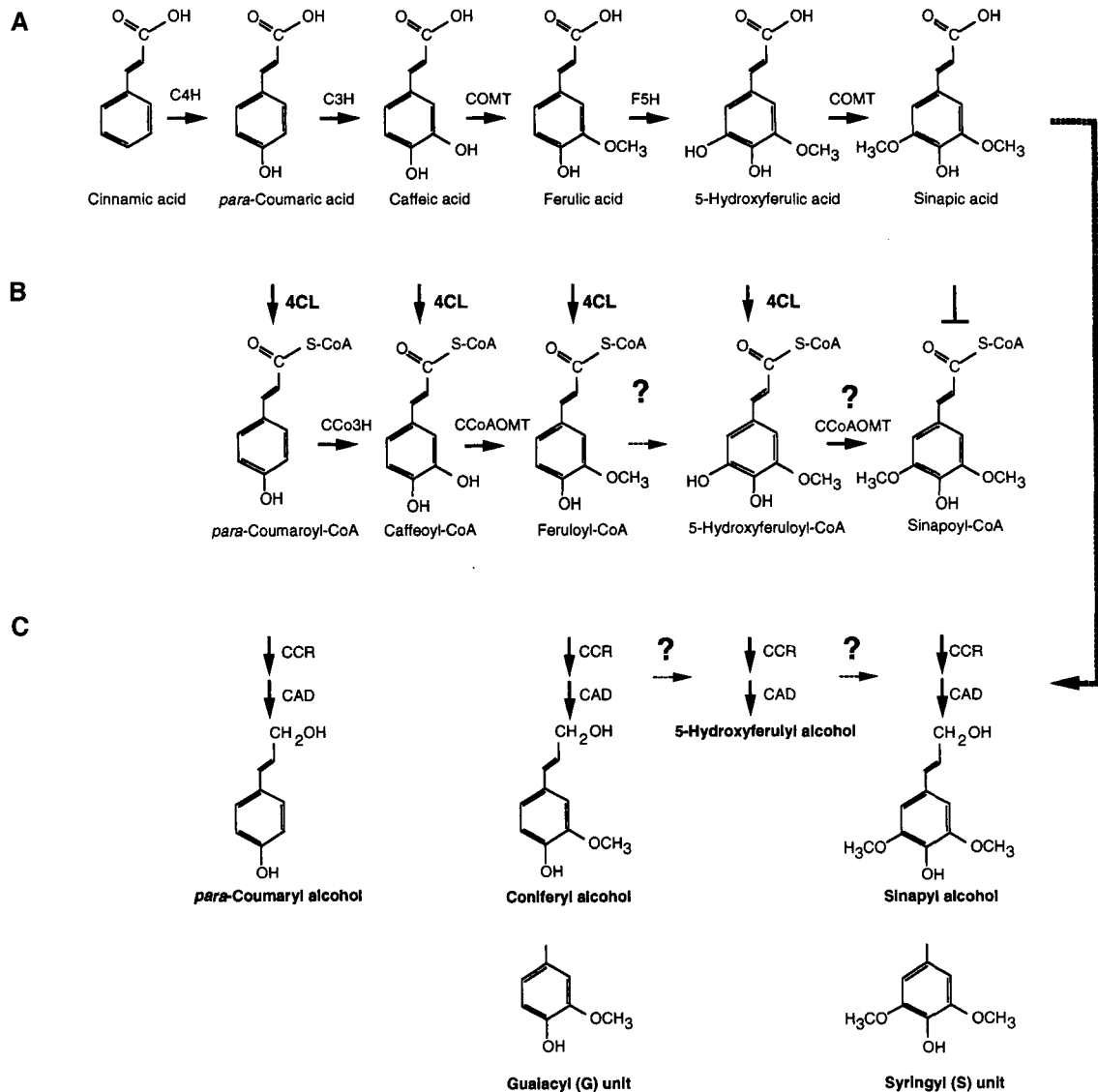


Figure 1. Overview of Metabolic Pathways Leading to Cinnamyl Alcohol Biosynthesis.

(A) Hydroxylation and methylation reactions at the free acid level, starting with cinnamic acid.

(B) Formation of CoA esters of hydroxycinnamic acids and hydroxylation and methylation reactions occurring at the CoA ester level.

(C) Reduction of CoA esters of hydroxycinnamic acids to cinnamyl alcohols and ring methylation patterns of G and S lignin subunits.

Solid arrows indicate enzymatic reactions catalyzed by the enzymes indicated. Dashed arrows indicate reactions that have not been demonstrated to occur or for which limited information is available. Question marks indicate reactions that may not occur in lignifying tissues, based on phenotypes of COMT antisense plants and the *fah1* (F5H) mutant. The long dashed arrow to the right of the diagram indicates a hypothetical 4CL-independent route to sinapyl alcohol. The upside-down T indicates lack of 4CL activity against sinapic acid in *Arabidopsis* stems and most other plants. CCo3H, coumaroyl-CoA-3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamyl-CoA reductase.

and stage of tissue development (Campbell and Sederoff, 1996). For example, the lignin in the tracheary elements of *Arabidopsis* vascular bundles is composed primarily of G units, whereas the adjacent highly lignified sclerenchyma, which differentiates later during stem development, contains

large amounts of syringyl units (Chapple et al., 1992). A major question in lignin biosynthesis regards the mechanisms that regulate differential carbon flow into the synthesis of these different lignin precursors, thus generating lignin polymers of differing subunit composition.

The phenotypes of transgenic tobacco and poplar plants with suppressed COMT activities suggest that a metabolic grid, as depicted in Figure 1, may function in the biosynthesis of coniferyl alcohol. Although the lignin of these COMT-suppressed plants is greatly depleted in S lignin subunits, it has correspondingly increased levels of G lignin subunits as well as unusual subunits based on 5-hydroxyferuloyl alcohol (Atanassova et al., 1995; Van Doorselaere et al., 1995). This indicates that either COMT or CCoOMT (Figures 1A and 1B) may be used in coniferyl alcohol biosynthesis. In contrast, COMT activity appears to be required for sinapyl alcohol biosynthesis, and apparently, sinapyl alcohol cannot be efficiently generated from feruloyl-CoA or its reduced derivatives in the lignifying stems of these plants via the alternative routes discussed above (Figures 1B and 1C). The Arabidopsis *fah1* mutant, which has a lesion in the F5H enzyme (Chapple et al., 1992; Meyer et al., 1996), is devoid of S lignin, again suggesting that feruloyl-CoA and its reduced derivatives are not efficiently diverted into sinapyl alcohol biosynthesis when sinapic acid biosynthesis is blocked. These results are most easily explained by the existence of partially separate pathways leading to the biosynthesis of coniferyl and sinapyl alcohols such that the potential metabolic grid leading to coniferyl alcohol does not extend to the biosynthesis of sinapyl alcohol.

The enzyme 4CL has been investigated in a large number of angiosperms, and in some plants, distinct isoforms of the enzyme with high activity against sinapic acid have been described (Knobloch and Hahlbrock, 1975; Ranjeva et al., 1976; Wallis and Rhodes, 1977; Grand et al., 1983). These isoforms could be involved in directing carbon flux into sinapyl alcohol; however, 4CL activity is usually low or non-detectable when sinapic acid is used as a substrate. This observation holds true for most angiosperms in which 4CL activity has been examined in crude extracts (summarized in Knobloch and Hahlbrock, 1977; Kutsuki et al., 1982; Lee and Douglas, 1996b), in purified or partially purified enzyme preparations (Gross and Zenk, 1974; Knobloch and Hahlbrock, 1977; Lozoya et al., 1988; A. Pri-Hadash, B. Ellis, and C.J. Douglas, unpublished data), or when heterologously expressed (Lee and Douglas, 1996b; S. Allina, A. Pri-Hadash, B. Ellis, D. Theilmann, and C.J. Douglas, manuscript submitted). Taken together, these data present an enigmatic picture of sinapyl alcohol biosynthesis: feruloyl-CoA and its reduced derivatives appear to be inefficiently converted into sinapyl alcohol, yet most plants do not appear capable of making sinapoyl-CoA directly via 4CL.

We previously identified an Arabidopsis *4CL* cDNA clone (Lee et al., 1995) and showed that the corresponding gene is highly expressed in Arabidopsis bolting stems, a major site of lignin deposition (Chapple et al., 1992; Dharmawardhana et al., 1992). We have now used an antisense approach to generate Arabidopsis lines with greatly reduced 4CL activity to clarify the role of 4CL in monolignol biosynthesis in this plant. Analysis of the lignin subunit composition in these lines, relative to wild-type plants, showed that whereas guai-

acyl lignin content was significantly reduced, there was no corresponding decrease in S units. This result suggests that 4CL is not required for sinapyl alcohol biosynthesis and implies the existence of a 4CL-independent pathway for the biosynthesis of this lignin precursor.

RESULTS

4CL Enzyme Activity

The 4CL enzymes in many plants have been shown to catalyze the biosynthesis of CoA esters from a variety of hydroxycinnamic acids, but no information is available for Arabidopsis. To examine the substrate specificity of Arabidopsis 4CL toward differently substituted cinnamic acid derivatives, we prepared crude protein extracts from bolting stems of Arabidopsis plants in which *4CL* is most highly expressed (Lee et al., 1995). Figure 2 shows that Arabidopsis 4CL had the highest activity toward 4-coumarate (4-hydroxycinnamate), the preferred substrate of most 4CL enzymes. Moderate levels of 4CL activity (10 to 50% of the activity toward 4-coumarate) were observed when ferulate (3-methoxy, 4-hydroxycinnamate), caffeate (3,4-

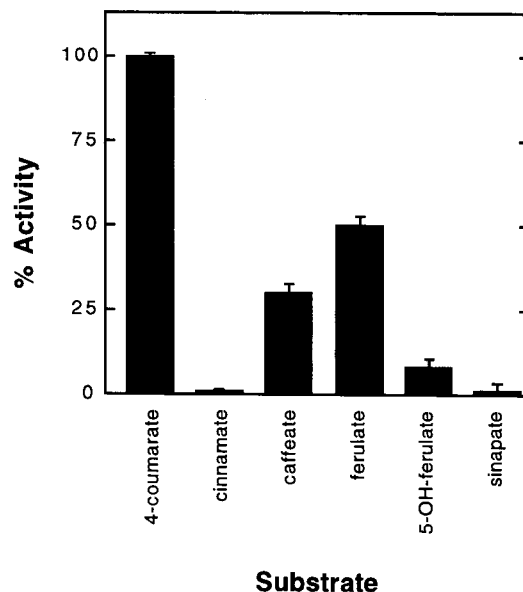


Figure 2. Utilization of Cinnamic Acid Derivatives by 4CL in Arabidopsis Stem Extract.

4CL activities in a protein extract from bolting stems were measured using the substrates indicated and expressed as a percentage of the activity against 4-coumarate. Error bars show the standard deviation from the mean of three replicate assays. The specific 4CL enzyme activity using 4-coumarate as a substrate (taken as 100%) was 145 μ kat/kg protein.

dihydroxycinnamate), and 5-hydroxyferulate were used as substrates.

The 4CL activities toward cinnamate and sinapate were very low (~1% of the activity toward 4-coumarate) and were at the limits of reproducible detection using the spectrophotometric assay employed. Similar results were observed when poplar and tobacco stem extracts were assayed for 4CL activity by using sinapate as a substrate (Lee and Douglas, 1996b; S. Allina, A. Pri-Hadash, B. Ellis, D. Theilmann, and C.J. Douglas, manuscript submitted). In these experiments, the production of sinapoyl-CoA could not be verified by HPLC, indicating that the spectrophotometric assay did not actually reflect sinapoyl:CoA formation by the *Arabidopsis* 4CL enzyme preparations. These results suggest that the biosynthesis of sinapoyl-CoA, thought to be the precursor for the biosynthesis of the sinapyl alcohol monomers of S lignin, does not proceed directly from sinapic acid in this plant.

Generation and Characterization of Antisense 4CL Lines

To gain insights into the metabolic function of the 4CL enzyme in lignin biosynthesis, we used an antisense RNA strategy to investigate the phenotypic consequences of suppressing 4CL expression. Approximately 1.6 kb of the *Arabidopsis* 4CL cDNA (Lee et al., 1995) was inserted behind either the parsley 4CL1 promoter or the cauliflower mosaic virus 35S promoter in antisense orientation, as shown in Figure 3. Antisense lines were generated by *Agrobacterium*-mediated transfer of the constructs into *Arabidopsis* RLD and Columbia ecotypes. A total of 27 transgenic RLD lines and eight transgenic Columbia lines were bred to homozygosity and used for further analysis.

To screen for transgenic lines with reduced 4CL protein levels, proteins were extracted from the bolting stems of wild-type and transgenic antisense 4CL plants and used on

immunoblots that were probed with an antibody raised against the parsley 4CL protein (Ragg et al., 1981). A representative blot is shown in Figure 4. Duplicate gels were run and stained with Coomassie blue to ensure uniform protein loading (not shown). More than 50% of the transgenic lines appeared to have lowered 4CL protein levels, and similar results were obtained with both the 4CL1 and 35S promoter-driven constructs.

Four antisense 4CL lines (out of a total of 32) that appeared to have the lowest levels of 4CL protein based on immunoblots were selected for detailed analysis. These lines included lines of both the RLD and Columbia (COL) ecotypes carrying the antisense *Arabidopsis* 4CL gene driven by either the parsley 4CL1 or 35S promoter. For simplicity, these transgenic lines are referred to as RLD:Pc4CL, RLD:35S, COL:Pc4CL, and COL:35S, respectively. DNA gel blot analysis demonstrated that COL:35S contained one copy of the antisense transgene, whereas RLD:Pc4CL, RLD:35S, and COL:Pc4CL contained one or more copies of the transgene (data not shown). The four transgenic *Arabidopsis* lines were normal in size, morphology, and fertility and had growth habits similar to those of wild-type plants. Figure 5 shows the morphology of the wild-type Columbia line (Figure 5A) and antisense Columbia lines (Figures 5B and 5C) at rosette and flowering stages (similar results were obtained for the RLD lines; data not shown).

The results of 4CL enzyme assays using *Arabidopsis* stem extracts, seen in Figure 6A, confirmed that the four transgenic lines had significantly reduced 4CL activities toward 4-coumaric acid, ranging from 8 to 50% residual 4CL enzyme activity compared with the activity in the corresponding wild-type plants. To determine whether 4CL activities against variously substituted cinnamic acid derivatives were also reduced in antisense 4CL lines, we assayed protein extracts from bolting stems of the most strongly reduced lines for 4CL activity by using these substrates. Table 1 shows

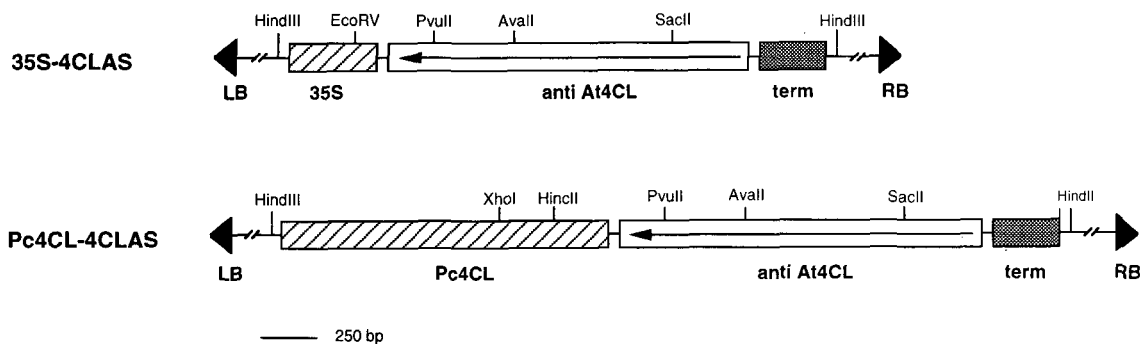


Figure 3. Structures of *Arabidopsis* 4CL Antisense Constructs 35S-4CLAS and Pc4CL-4CLAS.

Constructs are shown in their orientations with respect to the T-DNA left and right borders (LB and RB, respectively) in the binary vector pBin19. Hatched boxes, cauliflower mosaic virus 35S and parsley 4CL1 promoters; open boxes, *Arabidopsis* 4CL cDNA in antisense orientation with respect to the promoters; stippled boxes, nopaline synthase termination (*term*) sequences. Key restriction sites are shown.

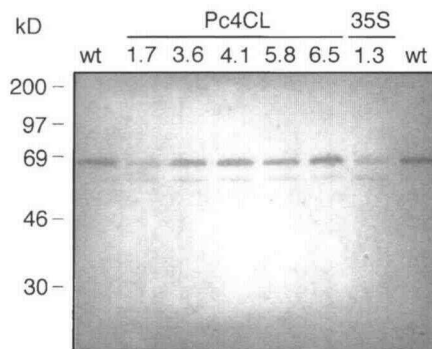


Figure 4. Example of an Immunoblot Used to Screen Transgenic Arabidopsis Plants.

Protein extracts are from stems of wild-type Columbia Arabidopsis (wt) or homozygous 4CL antisense lines (indicated by numbers) generated by *Agrobacterium*-mediated transfer of plasmid Pc4CL-4CLAS (Pc4CL) or 35S-4CLAS (35S). The blot was probed with the anti-4CL antiserum raised against parsley 4CL (Ragg et al., 1981). Molecular mass standards (in kilodaltons) are given at left.

that the residual 4CL activities against caffeate and ferulate were reduced proportionately with the decrease in 4CL activity toward 4-coumarate when they were measured using extracts from the same transgenic line. As in wild-type lines, the nominal sinapate-utilizing enzyme activity in the transgenic lines was very low and at the limit of reproducible detection using the spectrophotometric assay.

We next examined whether the block in 4CL expression in the antisense 4CL lines affected the expression of other genes in the phenylpropanoid pathway or in pathways of primary metabolism feeding into the phenylpropanoid pathway. To do this, we used RNA gel blots to analyze RNA accumulation after wounding of Arabidopsis leaves, a stimulus known to strongly activate phenylalanine ammonia-lyase (*PAL*), *C4H*, and 4CL expression (Ohl et al., 1990; Lee et al., 1995; Bell-Lelong et al., 1997; Mizutani et al., 1997).

Mature, fully expanded leaves from wild-type Columbia and the COL:Pc4CL and COL:35S antisense lines were wounded and then harvested for RNA extraction. Triplicate RNA gel blots were prepared and hybridized with probes corresponding to 4CL as well as genes encoding enzymes in the oxidative pentose phosphate pathway (6PGDH, 6-phosphogluconate dehydrogenase), the shikimic acid pathway (DHS1, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase), and the general phenylpropanoid pathway (*PAL* and *C4H*). In wild-type Columbia Arabidopsis plants, steady state 6PGDH, DHS1, EPSPS, *PAL*, *C4H*, and 4CL mRNA levels increased subsequent to wounding and reached maximal levels 1 to 2 hr after wounding (Figure 7).

In the 4CL-suppressed line COL:35S, no accumulation of 4CL transcripts was observed in response to wounding, whereas in line COL:Pc4CL, a small amount of 4CL transcript

was detected after 1 hr, consistent with the less severe repression of 4CL protein accumulation and 4CL enzyme activity in this line (Figures 5A and 6). Despite the strong suppression of 4CL expression in these transgenic lines, wound-induced accumulation of 6PGDH, DHS1, EPSPS, *PAL*, and *C4H* RNAs was identical to that observed in wild-type plants. Identical results were obtained with the wounded leaves of wild-type and antisense 4CL suppressed RLD lines of Arabidopsis (data not shown). Thus, 4CL expression in the antisense lines was strongly suppressed at the level of transcript accumulation as well as enzyme activity, but this suppression had no effect on the expression of the genes that are involved in 4-coumarate biosynthesis. In addition, immunoblot analysis using an antibody raised against parsley *PAL* (Schröder et al., 1976) showed that the transgenic lines had *PAL* protein levels that were the same as or slightly higher than the *PAL* protein levels found in wild-type Arabidopsis plants (data not shown). These results show that 4CL expression is specifically downregulated in the 4CL antisense plants.

Alteration of Lignin Content and Quality

To examine the effect of reduced 4CL activity on the accumulation of lignin, the thioglycolic acid (TGA) method (Bruce and West, 1989) was used to measure lignin content in stems of wild-type and antisense 4CL plants. The lignin content measured by this method was reduced in the antisense lines (Figure 6B). The degree of lignin reduction corresponded to the levels of residual 4CL enzyme activity found in the transgenic plants (cf. Figures 6A and 6B). Although reduction in lignin content followed the same pattern as reduction in 4CL enzyme activity, only the plants with >20% residual 4CL activity had lignin levels significantly less than those in wild-type plants.

Isolated cell walls from bolting stems were subjected to nitrobenzene oxidation (NBO) to determine whether any change in lignin subunit composition accompanied the decrease in lignin content in the antisense plants. Gas chromatography of NBO reaction products was used to quantify G lignin units (derived from feruloyl-CoA) as vanillin and vanillic acid and S units (thought to be derived from sinapoyl-CoA) as syringaldehyde and syringic acid (Figure 8). The ratio of G to S units (G/S value) measured in the bolting stems of wild-type lines was ~ 2.1 (Table 2), which is consistent with previous measurements (Chapple et al., 1992). However, there was a consistent and significant decrease in the quantity of G units by up to $\sim 40\%$ in the antisense plants (Table 2). In contrast, S unit content increased rather than decreased, leading to major increases in the mol% of S units and altering the G/S ratios in the antisense lines. Changes in the ratio correlated strictly with the extent of 4CL suppression (falling to 1.8, 1.15, 0.93, and 0.85 in antisense plants containing 49, 22, 16, and 8% residual 4CL activity, respectively); however, there was no consistent effect of 4CL suppression on the total NBO lignin content (sum of

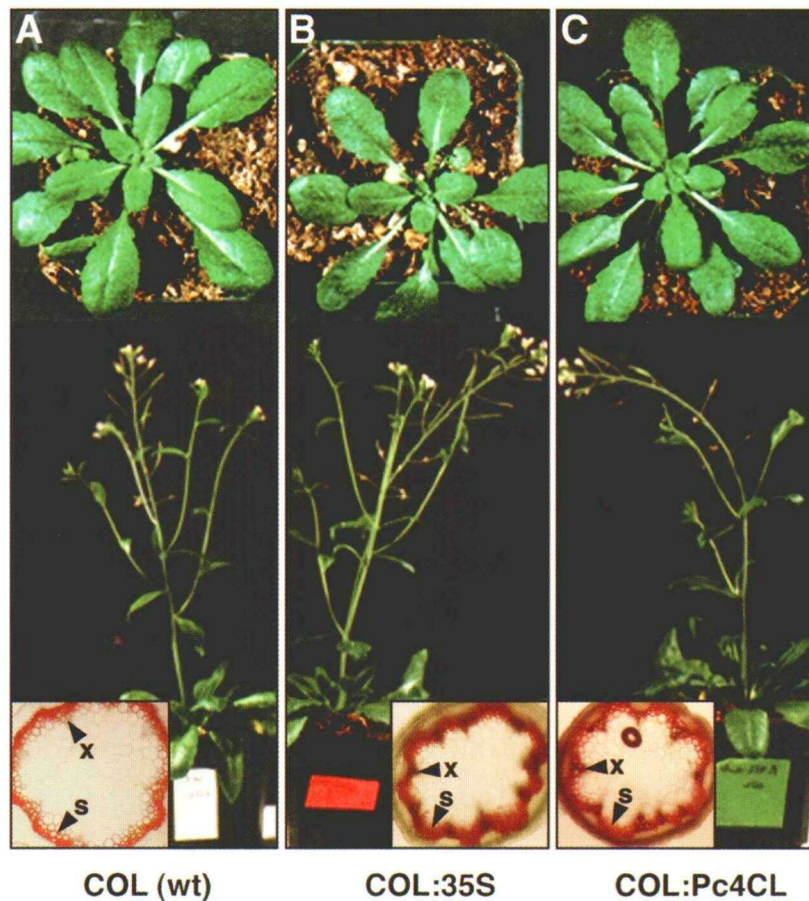


Figure 5. Morphology of Arabidopsis Columbia Antisense Lines COL:35S and COL:Pc4CL in Relation to Wild-Type Columbia Plants.

(A) Wild-type (wt) Columbia plants.

(B) Antisense line COL:35S.

(C) Antisense line COL:Pc4CL.

Rosette-stage plants are shown at the top; plants with bolting stems are shown at the bottom. Insets at the bottom show stem cross-sections stained with the Mäule reagent. s, sclerenchyma; x, xylem.

G plus S units). Histochemical staining of the lignified stem tissue using the Mäule reagent, which specifically stains syringyl units red, confirmed that the syringyl lignin content of the antisense 4CL plants was not greatly reduced relative to wild-type lines (Figure 5, insets). Thus, in Arabidopsis, it appears possible to increase the molar percentage of S units in the lignin of bolting stems, and thus decrease the G/S ratio of lignin monomers, in a predictable manner by decreasing 4CL enzyme activity levels.

DISCUSSION

The expression of transgenes for antisense 4CL in Arabidopsis proved to be effective in reducing 4CL activity by up

to 92% relative to activity in wild-type plants. Constructions based on the cauliflower mosaic virus 35S and the parsley 4CL1 promoters were comparable in their abilities to suppress 4CL expression. This was not unexpected because a 4CL1- β -glucuronidase (*GUS*) fusion has been shown to direct high levels of developmentally regulated and wound-inducible *GUS* expression in Arabidopsis, similar to that of the Arabidopsis 4CL gene (Lee et al., 1995). The level of 4CL suppression we obtained is comparable to that achieved for other phenylpropanoid and lignin-related genes by sense or antisense suppression. *PAL* sense-suppressed tobacco lines show up to 95% lower levels of *PAL* activity (Elkind et al., 1990), antisense *CAD* tobacco plants have up to 93% decrease in *CAD* activity (Halpin et al., 1994), and tobacco and poplar plants with transgenes antisense to *COMT* have residual *COMT* activities as low as 2 to 5% relative to control

plants (Atanassova et al., 1995; Van Doorselaere et al., 1995).

The Arabidopsis lines with reduced 4CL activity were morphologically normal but had distinct phenotypes with respect to the lignin content in bolting stems. The maximum reduction in TGA extractable lignin content achieved was ~50% relative to wild-type levels. Statistically significant decreases in lignin content were not observed until residual 4CL activity decreased to >20% of wild-type levels (Figure 6). Similar results were reported in *PAL*-suppressed tobacco plants, where *PAL* activity only becomes rate limiting for lignin accumulation (also estimated by TGA extraction) when enzyme activity is decreased by 75 to 80% relative to wild-type levels (Bate et al., 1994). In contrast to our experiments, however, it was possible to reduce lignin content in the *PAL*-suppressed plants to as low as 10% of that found in control plants (Bate et al., 1994).

When lignin content in the antisense 4CL lines was estimated by the sum of the G and S units generated by NBO, there was little if any change relative to wild-type plants (Table 2). The discrepancy between these results and those obtained by TGA extraction likely reflects the different mechanisms by which these procedures extract lignin. Treatment with TGA extracts a lignothiolyglycolic acid complex from cell walls (Whitmore, 1978; Campbell and Ellis, 1992), whereas NBO releases a subset of lignin monomers that comprise uncondensed units devoid of aromatic carbon-carbon linkages (Lewis and Yamamoto, 1990). Thus, changes in lignin extractability, but not actual content, could lead to apparent changes in lignin content as measured by TGA. Alternatively, if changes in the amount of lignin in the antisense lines were in condensed regions of the polymer, NBO could underestimate the change in lignin content in these lines.

Although more extensive characterization of the lignin in the antisense lines is required to resolve these issues, it is apparent that even the most highly suppressed 4CL lines exhibit at most fairly modest changes in total lignin content. This was confirmed by staining with phloroglucinol and Mäule reagents, which failed to distinguish between the lignin in wild-type and antisense lines (Figure 5 and data not shown). Thus, our data could indicate that the 4CL activity level in bolting stems is very much in excess of that required for the developmentally regulated biosynthesis lignin monomers under laboratory conditions, so that even larger reductions in residual 4CL activity would be required to further reduce lignin content. Alternatively, the data could be explained by the existence of one or more alternate pathways for lignin monomer biosynthesis in Arabidopsis that operate independently of 4CL activity. In this case, the carbon flux through such a 4CL-independent pathway(s) would be unaltered in the antisense 4CL lines, whereas that in the 4CL-dependent pathway would be reduced, leading to moderate decreases in lignin content. For reasons outlined below, we favor the latter explanation.

First, the biosynthetic origin of sinapyl alcohol, and the role played by 4CL in its biosynthesis, is unclear. The apparent

absence of 4CL activity toward sinapate in Arabidopsis bolting stems (Figures 1 and 2), in which large amounts of S lignin accumulate (Chapple et al., 1992; Dharmawardhana et al., 1992), suggests that as in tobacco (Lee and Douglas, 1996b), poplar (S. Allina, A. Pri-Hadash, D. Theilmann, B. Ellis, and C.J. Douglas, manuscript submitted), and most other angiosperms, sinapoyl-CoA is not synthesized from sinapic acid through the 4CL catalyzed reaction (Figure 1A). Another possible route would be the alternate pathway through which sinapoyl-CoA could be synthesized by consecutive hydroxylation and methylation of feruloyl-CoA (Figure 1B).

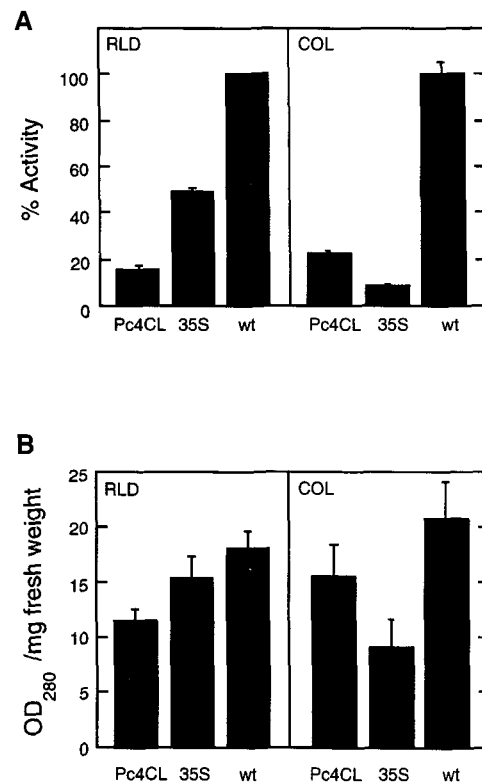


Figure 6. 4CL Enzyme Activity and Lignin Content in 4CL Antisense and Wild-Type Arabidopsis Stems.

(A) 4CL activity in protein extracts from bolting stems. Enzyme activity in four antisense lines RLD:Pc4CL, RLD:35S, COL:Pc4CL, and COL:35S is expressed as a percentage of the activity in untransformed (wild-type [wt]) RLD or Columbia plants, respectively. Results are averaged from three determinations; error bars represent standard deviations.

(B) Estimation of lignin content in 4CL antisense and wild-type Arabidopsis. Lignin was extracted from the bolting stems of wild-type RLD or Columbia plants (wt) and the four antisense lines RLD:Pc4CL, RLD:35S, COL:Pc4CL, and COL:35S by using the TGA method. Relative lignin amounts are expressed as the optical density at 280 nm. Results are averaged from three determinations, and error bars represent standard deviations.

Table 1. 4CL Activity against Differently Substituted Hydroxycinnamic Acids in Arabidopsis Wild-Type and 4CL Antisense Lines

| Line | Specific Activity ($\mu\text{kat}/\text{kg}$ protein) Using a Given Substrate ^a | | | | |
|---------------|---|-----------------|----------------|----------------|---------------|
| | Coumarate | Cinnamate | Caffeate | Ferulate | Sinapate |
| RLD wild type | 98.7 | 0.9 | 34.4 | 41.2 | 4.9 \pm 6.4 |
| RLD:Pc4CL | 13.9 | ND ^b | 13.8 | 7.7 | 2.3 |
| COL wild type | 145.4 \pm 0.9 ^c | 0.7 \pm 0.4 | 43.7 \pm 4.4 | 72.0 \pm 3.5 | 1.7 \pm 3.0 |
| COL:35S | 21.5 \pm 3.3 | ND | 14.3 \pm 6.4 | 18.5 \pm 8.9 | 6.5 \pm 3.9 |

^a Values are based on one determination, except where standard deviations are given in which the values are the mean of three assays.

^b ND, no activity detected.

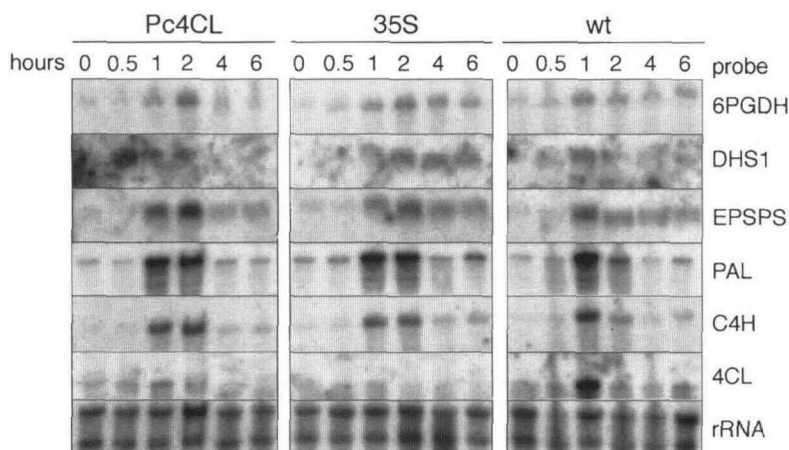
^c Mean of three determinations \pm SD.

This biosynthetic route would avoid the necessity of 4CL activity against sinapate to generate sinapoyl-CoA (Ye et al., 1994). However, the phenotypes of transgenic tobacco and poplar plants with suppressed COMT activities (Atanassova et al., 1995; Van Doorselaere et al., 1995) and the Arabidopsis *fah1* (Chapple et al., 1992) mutant lacking F5H activity argue against the operation of such alternate routes to sinapyl alcohol in lignifying stem tissues. In the absence of 4CL activity against sinapic acid in lignifying tissues of Arabidopsis, tobacco, and poplar, these results leave the biosynthetic origin(s) of sinapyl alcohol unresolved. They suggest, however, the existence of distinct pathways for coniferyl and sinapyl alcohol biosynthesis, consistent with the possible existence of a 4CL-independent biosynthetic route to sinapyl alcohol.

Second, the most striking biochemical phenotype of the Arabidopsis antisense 4CL lines is a consistent and predict-

able decrease in the G/S ratio of lignin in bolting stems, as measured by NBO. As discussed above, NBO only samples the monomeric composition of the noncondensed portions of the lignin polymer, in which aromatic moieties are devoid of intercarbon linkages (Lewis and Yamamoto, 1990); however, it provides an internally consistent measure of lignin monomer composition within a given species. The decreases in the G/S ratio in the 4CL antisense plants are brought about by significant decreases in G lignin units and corresponding increases in S lignin units (Table 2). The decreases in the G/S ratio were in turn tightly correlated to residual 4CL activity levels (Table 2).

Additional evidence for the continued accumulation of S units in the lignin of the 4CL antisense lines comes from the deep red staining of lignified cells in these plants with the Mäule reagent, a reaction diagnostic for the presence of S

**Figure 7.** Wound-Induced RNA Accumulation in Leaves of Wild-Type and 4CL Antisense Arabidopsis Plants.

Gel blots of RNA extracted from wounded leaves of wild-type (wt) Columbia plants and antisense lines COL:Pc4CL (Pc4CL) and COL:35S (35S) were hybridized with the probes indicated at right. Mature, fully expanded leaves were wounded and incubated for 0, 0.5, 1, 2, 4, and 6 hr before RNA extraction. RNA samples (10 μg) were fractionated in triplicate on formaldehyde gels, transferred to nylon membranes, and hybridized with DNA probes from Arabidopsis cDNAs encoding 6PGDH, DHS1, EPSPS, PAL, and C4H. A single-stranded antisense RNA probe was used to specifically detect endogenous 4CL transcripts. Hybridization of each blot with an rRNA probe demonstrated uniformity of RNA loading; the blot previously hybridized to PAL, C4H, and 4CL probes is shown as an example (rRNA).

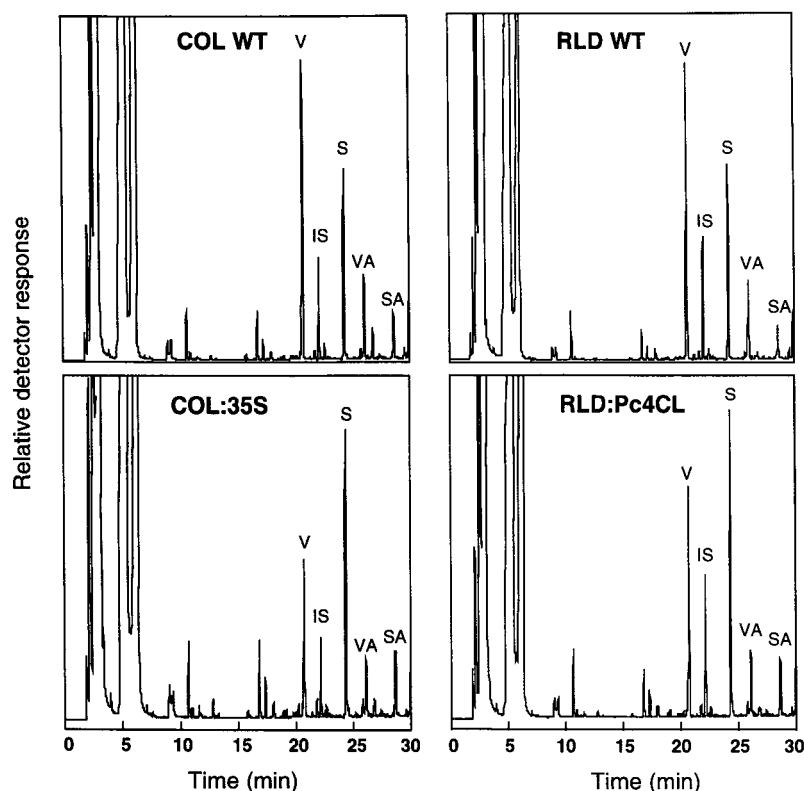


Figure 8. Gas Chromatography of NBO Products from Wild-Type and 4CL Antisense Lines.

Representative chromatograms from wild-type Columbia (COL WT) and RLD (RLD WT) lines and two 4CL antisense lines (COL:35S and RLD:Pc4CL) are shown. Peaks corresponding to syringaldehyde (S), syringic acid (SA), vanillin (V), and vanillic acid (VA) are labeled. IS indicates the position of the internal standard, 3-ethoxy-4-hydroxybenzaldehyde.

units (Lewis and Yamamoto, 1990). Thus, partial blocking of 4CL activity has opposite effects on the accumulation of G and S lignin monomers. The most likely explanation for this finding is reduced carbon flux through the pathway leading to coniferyl alcohol but unaltered or increased flux through a

pathway(s) leading to sinapyl alcohol in the antisense lines (see Figure 1). This supports the accepted role for 4CL in the biosynthesis of coniferyl alcohol and guaiacyl lignin but strongly suggests that 4CL activity is not required for the biosynthesis of sinapyl alcohol and S lignin. Coupled with

Table 2. Analysis of Lignin Monomer Composition in Arabidopsis Wild-Type and 4CL Antisense Lines by Nitrobenzene Oxidation and Gas Chromatography

| Line | % 4CL Activity | G Units ^a ($\mu\text{mol g}^{-1}$ dw) | S Units ^b ($\mu\text{mol g}^{-1}$ dw) | G + S Units ($\mu\text{mol g}^{-1}$ dw) | G/S |
|-----------|----------------------------|--|--|---|------|
| RLD | 100 \pm 0.2 ^c | 6.61 \pm 0.62 | 3.19 \pm 0.20 | 9.80 \pm 0.72 | 2.1 |
| RLD:35S | 48.8 \pm 1.9 | 7.14 \pm 0.94 | 3.94 \pm 0.93 | 11.1 \pm 0.93 | 1.8 |
| RLD:Pc4CL | 15.6 \pm 1.5 | 4.01 \pm 0.68 | 4.31 \pm 0.99 | 8.32 \pm 1.64 | 0.93 |
| Columbia | 100 \pm 5.2 | 5.20 \pm 0.45 | 2.56 \pm 0.52 | 7.76 \pm 0.60 | 2.0 |
| COL:Pc4CL | 21.9 \pm 0.2 | 3.16 \pm 0.13 | 2.77 \pm 0.49 | 5.93 \pm 0.60 | 1.15 |
| COL:35S | 8.4 \pm 0.6 | 3.93 \pm 0.56 | 4.59 \pm 0.45 | 8.52 \pm 0.75 | 0.85 |

^a Sum of vanillin plus vanillic acid, as determined by gas chromatography. dw, dry weight.

^b Sum of syringaldehyde plus syringic acid, as determined by gas chromatography.

^c Relative 4CL activities from Figure 6A; NBO data are from the mean of four replicates \pm SD.

the lack of measurable 4CL activity against sinapic acid in *Arabidopsis* and most other plants, the simplest explanation for these results is the existence of an uncharacterized pathway for the biosynthesis of sinapyl alcohol and S lignin, independent of 4CL activity (Figure 1; see below).

The results of a recent investigation of the effect of suppressed 4CL activity on the lignin content of transgenic tobacco plants (Kajita et al., 1996) are consistent with many of our observations in *Arabidopsis*. In that study, sense and antisense 4CL suppression led to residual 4CL activities as low as 1% of control levels. However, the total lignin content in the most severely suppressed plants was 65 to 78% of control values, and the morphology of the plants was apparently normal. These results confirm that, unlike suppression of PAL activity (Bate et al., 1994), near total blockage of 4CL activity only modestly affects total lignin content and has little effect on the development of the vascular system. Tobacco plants with suppressed 4CL activities had spatially separated sectors with lignin polymers of distinct subunit composition. In several antisense plants, and within the abnormal sectors of one plant examined in detail, there were dramatic increases in S units relative to G units, as measured by NBO (Kajita et al., 1996), although the lignin G/S ratio in these tobacco plants was not always closely related to the residual 4CL activity levels. Similar to *Arabidopsis* stems (Figure 2), 4CL activity from crude tobacco stem extracts reacts minimally, if at all, against sinapate (Lee and Douglas, 1996b). These results from tobacco also support the existence of an alternative, 4CL-independent pathway for S monomer biosynthesis.

Other explanations might be invoked to account for the altered G/S ratios in 4CL antisense lines. Differential suppression of the activity of divergent *Arabidopsis* 4CL isoforms, if such exist, could in theory result in lines with altered 4CL substrate utilization profiles. This might be particularly relevant to tobacco, where multiple 4CL genes encoding divergent 4CL isoforms are present (Lee and Douglas, 1996b). Our earlier data suggested that 4CL is encoded by a single gene in *Arabidopsis*, represented by the At4CL cDNA (Lee et al., 1995), and so far cDNA clones essentially identical to At4CL are the only sequences with strong similarity to 4CL as represented in the *Arabidopsis* expressed sequence tag database (Newman et al., 1994). Thus, 4CL isoforms with different substrate specificity and assay requirements may not exist in *Arabidopsis*, though this possibility cannot be completely ruled out at present.

We have also demonstrated that the genes encoding representative enzymes of the oxidative pentose phosphate (6PGDH), shikimic acid (DAHP synthase and EPSP synthase), and general phenylpropanoid (PAL, C4H, and 4CL) pathways are coordinately activated in response to wounding stress in *Arabidopsis*. These results are consistent with those obtained in elicitor-treated alfalfa (Fahrendorf et al., 1995), tomato (Görlach et al., 1995), and parsley (Logemann et al., 1995) cell cultures but to our knowledge represent the first report in a single species in which genes in all three pathways have been shown to be coordinately activated.

Our results underscore the fact that activation of phenylpropanoid product biosynthesis is regulated in concert with activation of pathways of primary metabolism to supply the carbon precursors and reducing equivalents used in phenylpropanoid natural product biosynthesis. Furthermore, our work demonstrates that a significant block in 4CL expression does not affect expression of these other representative genes in these pathways (Figure 7), consistent with evidence in other plants (Fahrendorf et al., 1995; Görlach et al., 1995) that such pathways are direct targets of stress-activated signaling mechanisms.

These data are important because suppression of 4CL activity could theoretically lead to secondary effects on the expression of other genes related to lignin biosynthesis, due to reduced metabolic flux through the pathway and increased pool sizes of metabolic intermediates. Cinnamic acid, which could increase in pool size in 4CL-suppressed plants, has been shown to repress expression of PAL genes (Mavandad et al., 1990). Furthermore, suppression of PAL and C4H activity can result in deposition of lignin with altered subunit composition (Sewalt et al., 1997). However, in the *Arabidopsis* lines described here, the patterns of expression of a suite of genes encoding enzymes metabolically upstream of 4CL were identical to the expression patterns in wild-type plants (Figure 7); therefore, the partial block in 4CL activity does not affect the expression of these genes. In addition, immunoblots demonstrated that PAL protein levels were not reduced in the antisense lines (data not shown). Thus, the lignin phenotype of the antisense 4CL plants cannot easily be attributed to changes in gene expression indirectly brought about by the block in 4CL expression.

What might be an alternative pathway for the reduction of sinapic acid to S alcohol? Thermodynamic considerations make it unlikely that free hydroxycinnamic acids are themselves reduced to their corresponding cinnamaldehydes, which are further reduced to alcohols (monolignols), but rather that activated carboxylic acid intermediates such as CoA thioesters are utilized in these cellular reactions (Grisebach, 1981). After the demonstration that CoA esters are formed by the action of 4CL (Hahlbrock and Grisebach, 1970), cinnamyl alcohols were shown to be formed via these esters (Mansell et al., 1972; Ebel and Grisebach, 1973). Furthermore, 4CL expression patterns are tightly correlated with sites of lignin deposition (Hauffe et al., 1991). Thus, there is little doubt that hydroxycinnamoyl-CoA esters formed by the action of 4CL are intermediates in the biosynthesis of at least some cinnamyl alcohols. Our data suggest, however, that it is primarily coniferyl alcohol, derived by reduction of ferulic acid, that is made via cinnamoyl-CoA esters. The sinapic acid-derived intermediate that serves as the activated intermediate for reduction to sinapaldehyde and sinapyl alcohol remains to be identified.

Attempts to use genetic engineering to modify lignin deposition in trees have focused on the reduction of total lignin content and/or the alteration of its subunit composition (Boudet and Grima-Pettanati, 1996; Campbell and Sederoff,

1996; Douglas, 1996). The modification of lignin monomer content is of particular interest because lignin with high S subunit composition can be more efficiently extracted during the kraft pulping process. Our results in Arabidopsis, and those of Kajita et al. (1996) in tobacco, suggest that suppression of 4CL activity may be an effective way both to modestly decrease the lignin content of wood and to shift the G/S subunit ratio of this lignin in favor of syringyl units. The apparent existence of a 4CL-independent pathway for sinapyl alcohol biosynthesis may also be relevant to the biotechnological introduction of S lignin biosynthesis into conifers, which are devoid of this lignin subunit.

METHODS

Plant Growth Conditions and Transformation

Plants (*Arabidopsis thaliana*) were grown in soil (Fisons Sunshine Mix; Sun Gro Horticulture Inc., Bellevue, WA) in growth chambers at 23°C under $\sim 120 \mu\text{E sec}^{-1} \text{m}^{-2}$ light in an 8 hr dark/16 hr light regime. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated root transformation (Valvekens et al., 1988; Lee and Douglas, 1996a). The primary transformants (T_0) were allowed to mature and set seed in tissue culture at 23°C under $\sim 150 \mu\text{E m}^{-2} \text{sec}^{-1}$ of constant light. T_1 seedlings from each transgenic line were selected for growth on 50 mg/L kanamycin and then allowed to self and set seed after transfer to soil. Seed lots from T_1 plants (T_2 lines) that gave rise to 100% kanamycin-resistant seedlings were thus likely to be homozygous lines and were used for subsequent analyses.

Plant Tissues and Treatments

Enzyme assays, immunoblots, and lignin extractions were performed using Arabidopsis bolting stems that were 15 cm in height, excluding secondary inflorescences, bracts, flowers, and developing siliques. Mature, fully expanded Arabidopsis leaves were wounded by slicing the leaves into 1- to 2-mm-wide strips. The wounded leaves were placed on a piece of filter paper moistened with Murashige and Skoog medium (Gibco BRL) before harvest into liquid nitrogen. For 0 hr of wounding, leaves were detached from the plants and immediately frozen in liquid nitrogen.

DNA Constructions

Antisense DNA constructs were prepared using pRT101, a cloning vector containing a 0.4-kb fragment of the cauliflower mosaic virus 35S promoter (Töpfer et al., 1987) or a derivative (pRT101-Pc4CL). pRT101-Pc4CL was generated by replacing the 35S promoter in pRT101 with a 1.5-kb PstI-EcoRI fragment of the parsley 4CL1 promoter (Hauffe et al., 1991). To prepare the Arabidopsis 4CL antisense construct under the control of the 35S promoter (35S-4CLAS), a 1.6-kb XbaI-BglII fragment of the Arabidopsis 4CL cDNA (At4CL; Lee et al., 1995) containing the complete coding region but missing most of the 3' untranslated region was cloned into the BamHI-XbaI site of pRT101 in the antisense orientation. To prepare the Arabidopsis 4CL antisense construct under the control of the 4CL1 promoter (Pc4CL-

4CLAS), the 1.6-kb XbaI-BglII At4CL fragment was cloned into the BamHI-XbaI site of pRT101-Pc4CL in the antisense orientation. Restriction sites derived from the pBluescript KS+ vector (Stratagene, La Jolla, CA) harboring the original 4CL cDNA clone were removed by digesting the DNA constructs with XbaI and ClaI, generating blunt-end termini by using the Klenow fragment of DNA polymerase I, and religating the plasmids. Both DNA constructs were then subcloned as HindIII cassettes into the HindIII site of the Agrobacterium binary vector pBin19 (Bevan, 1984).

Immunoblot Analysis

Protein extracts were prepared by grinding the plant tissue in liquid nitrogen and resuspending the powder in 50 mM Tris, pH 8, and 5 mM MgCl_2 . The homogenate was centrifuged twice at 4°C to remove cellular debris, and the final supernatant was used for immunoblot analysis. Protein concentrations were determined using the BioRad protein assay kit with BSA as a standard. Protein samples (25 μg) were run on 10% SDS-PAGE gels (Laemmli, 1970) and blotted onto Hybond (Amersham, Arlington Heights, IL) nylon membranes at 4°C for 3 hr by using transfer buffer (25 mM Tris-HCl and 250 mM glycine). Blots were blocked with 5% nonfat powdered milk, reacted with antibodies raised against parsley 4CL (Ragg et al., 1981) or parsley PAL (Schröder et al., 1976), reacted with an anti-rabbit secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase; Gibco BRL, Gaithersburg, MD), and then visualized using 4.5 mM Fast-Red R and 2.5 mM naphthol AS-MX Phosphate (Sigma) dissolved in 50 mM Tris, pH 8, as substrates. SDS-PAGE gels using identical amounts of extracts were run in parallel and stained with Coomassie Brilliant Blue R 250 to assess uniformity of protein loading.

4CL Enzyme Assay

Extracts were prepared as described by Knobloch and Hahlbrock (1977). Briefly, plant tissues were ground into a fine powder in liquid nitrogen, resuspended in 200 mM Tris, pH 7.8, and 15 mM β -mercaptoethanol, and rotated in the presence of 10% Dowex AG 1-X2 Resin (Bio-Rad) at 4°C for 15 min. The mixture was centrifuged twice to remove debris, and the supernatant was made to 30% glycerol, frozen in liquid nitrogen, and stored at -80°C . 4CL enzyme activity was measured spectrophotometrically at room temperature, as described by Knobloch and Hahlbrock (1977). The 4CL reaction mixtures contained protein extracts, 5 mM ATP, 5 mM MgCl_2 , 0.33 mM CoA, and 0.2 mM cinnamic acid derivatives. The blank (reference) mixtures contained the same components but without CoA. Enzyme activity was measured as the increase in absorbance at the absorption maximum of the appropriate CoA ester, shown in Table 3. The extinction coefficients of these esters (Table 3) were used to calculate enzyme activities.

RNA Gel Blot Analysis

RNA extraction and preparation of RNA gel blots were as described by Lee and Douglas (1996b), except that Zeta probe GT membranes (Bio-Rad) were used. Hybridizations and stripping of blots were performed as specified by the manufacturer. All blots were washed at high stringency (0.2 \times SSC [1 \times SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS, at 65°C). Phosphorus-32-radiolabeled

Table 3. Absorption Maxima and Extinction Coefficients Used to Assay and Calculate 4CL Activities

| Product | Absorption Maximum (nm) | Extinction Coefficient (L mol ⁻¹ cm ⁻¹) | Reference |
|-------------------|-------------------------|--|--|
| Cinnamoyl-CoA | 311 | 22,000 | Gross and Zenk (1966) |
| 4-Coumaroyl-CoA | 333 | 21,000 | Stöckigt and Zenk (1975) |
| Caffeoyl-CoA | 346 | 18,000 | Stöckigt and Zenk (1975) |
| Feruloyl-CoA | 345 | 19,000 | Gross and Zenk (1966) |
| 5-OH-feruloyl-CoA | 356 | 20,000 | Knobloch and Hahlbrock (1975); Lüderitz et al. (1982) |
| Sinapoyl-CoA | 352 | 20,000 | Lüderitz et al. (1982) |

DNA probes (random primers DNA labeling system; Gibco BRL) were generated from the following sources. The *PAL* probe was made using a HindIII fragment from the Arabidopsis *PAL 10-3* gene corresponding to a conserved portion of Arabidopsis *PAL* genes (Wanner et al., 1995). The *DSH1* probe was generated from the Arabidopsis *DSH1* cDNA (Keith et al., 1991). The C4H probe was generated from an Arabidopsis C4H cDNA clone (Bell-Lelong et al., 1997). cDNA clones encoding 6PGDH and EPSPS were obtained by keyword searching of the Arabidopsis expressed sequence tag database (Newman et al., 1994). Arabidopsis expressed sequence tag 11B4T7, the 5' end of which is ~80% identical to the 6PGDH cloned from alfalfa, was used to generate the 6PGDH probe. Expressed sequence tag 131D24T7, the 5' end of which is 98% identical to the Arabidopsis EPSPS gene, was used to generate the EPSPS probe. The sequences of these expressed sequence tag clones were verified before use. The rRNA probe was generated from a HindIII fragment of a pea rRNA gene (Jorgensen et al., 1982). A phosphorus-32-radiolabeled single-stranded antisense 4CL RNA probe was generated from the Arabidopsis 4CL cDNA At4CL (Lee et al., 1995).

Lignin Analysis

Thioglycolic acid extraction of lignin was performed as described by Bruce and West (1989), using methanol-extracted cell wall material from ~50 mg (wet weight) Arabidopsis stem samples. Approximately 10-mg samples of air-dried methanol insoluble cell wall material were used for thioglycolic acid extraction of lignin, as described by Bruce and West (1989). Histochemical staining using the Mäule reagent was performed as described by Chapple et al. (1992).

For the determination of lignin monomer composition by nitrobenzene oxidation (NBO), stem tissue of mature, 5-week-old Arabidopsis plants grown at 22°C with a 16-hr light/8-hr dark photoperiod were ground to a powder in liquid nitrogen and extracted with 20 mL of 0.1 M sodium phosphate buffer, pH 7.2, at 37°C for 30 min followed by three extractions with 80% ethanol at 80°C. The tissue was then extracted once with acetone and dried. Tissue was saponified by treatment with 1.0 M NaOH at 37°C for 24 hr, washed three times with water, once with 80% ethanol, once with acetone, and dried.

NBO of stem tissue samples was performed with a protocol modified from Iiyama and Lam (1990). Samples of lignocellulosic material (5 mg each) were mixed with 500 μ L of 2 M NaOH and 25 μ L of nitrobenzene. This mixture was incubated in a sealed glass tube at 160°C for 3 hr. The reaction products were cooled to room temperature, and 5 μ L of a 20 mg mL⁻¹ solution of 3-ethoxy-4-hydroxybenzaldehyde in pyridine was added as an internal standard before the mixture was extracted twice with 1 mL of dichloromethane. The

aqueous phase was acidified with HCl, pH 2, and extracted twice with 900 μ L of ether. The combined ether phases were dried with anhydrous sodium sulfate, and the ether was evaporated in a stream of nitrogen. The dried residue was resuspended in 50 μ L of pyridine, 10 μ L of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide) was added, and 1- μ L aliquots of the silylated products were analyzed using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Supelco (Bellefonte, PA) SPB I column (30 m \times 0.75 mm). Lignin monomer composition was calculated from the integrated areas of the peaks representing the trimethylsilylated derivatives of vanillin, syringaldehyde, vanillic acid, and syringic acid. Total NBO-susceptible G units (vanillin and vanillic acid) and S units (syringaldehyde and syringic acid) were calculated after correction for recovery efficiencies of each of the products during the extraction procedure relative to the internal standard.

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