

Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.)

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Summary

Genes encoding seven enzymes of the monolignol pathway were independently downregulated in alfalfa (*Medicago sativa*) using antisense and/or RNA interference. In each case, total flux into lignin was reduced, with the largest effects arising from the downregulation of earlier enzymes in the pathway. The downregulation of L-phenylalanine ammonia-lyase, 4-coumarate 3-hydroxylase, hydroxycinnamoyl CoA quinate/shikimate hydroxycinnamoyl transferase, ferulate 5-hydroxylase or caffeic acid 3-O-methyltransferase resulted in compositional changes in lignin and wall-bound hydroxycinnamic acids consistent with the current models of the monolignol pathway. However, downregulating caffeoyl CoA 3-O-methyltransferase neither reduced syringyl (S) lignin units nor wall-bound ferulate, inconsistent with a role for this enzyme in 3-O-methylation of S monolignol precursors and hydroxycinnamic acids. Paradoxically, lignin composition differed in plants downregulated in either cinnamate 4-hydroxylase or phenylalanine ammonia-lyase. No changes in the levels of acylated flavonoids were observed in the various transgenic lines. The current model for monolignol and ferulate biosynthesis appears to be an over-simplification, at least in alfalfa, and additional enzymes may be needed for the 3-O-methylation reactions of S lignin and ferulate biosynthesis.

Keywords: cell wall phenolics, genetic modification, lignification, O-methylation, transgenic alfalfa.

Introduction

Lignin is a complex polymer of hydroxylated and methoxylated phenylpropane units, linked via oxidative coupling (Boudet *et al.*, 1995), and accounts for some 30% of the organic carbon in the biosphere. It is important for structural integrity of the plant cell wall and the mechanical properties and physical strength of stems (Chabannes *et al.*, 2001; Jones *et al.*, 2001). Lignin from angiosperms usually contains two major monolignols, 4-hydroxy, 3-methoxy substituted guaiacyl (G) units and 4-hydroxy, 3,5-dimethoxy substituted syringyl (S) units, linked by at least five different types of linkages (Davin and Lewis, 1992).

Genetic and transgenic studies have questioned the true *in vivo* specificities of the monolignol pathway enzymes as initially extrapolated from *in vitro* studies (Chen *et al.*, 1999; Franke *et al.*, 2002; Li *et al.*, 2000; Schoch *et al.*, 2001; Zhong *et al.*, 1998). None of the ring

substitution steps beyond the 4-hydroxylation reaction take place at the level of the free acid in the currently accepted pathway. Rather, 3-hydroxylation occurs at the level of a shikimate ester, methylation of the 3-hydroxyl occurs at the level of a coenzyme A ester, and 5-hydroxylation occurs at either the aldehyde or alcohol level (Figure 1). Furthermore, genetic evidence in Arabidopsis suggests that ferulate, a common component of plant cell walls, is not formed by the methylation of caffeic acid, as thought previously, but rather from coniferaldehyde by the action of an aldehyde dehydrogenase (ALDH, Figure 1) (Nair *et al.*, 2004). This suggests that the methyl group in ferulate is introduced by caffeoyl coenzyme A 3-O-methyltransferase (CCoAOMT), rather than by caffeic acid 3-O-methyltransferase (COMT), which is currently viewed as functioning downstream in

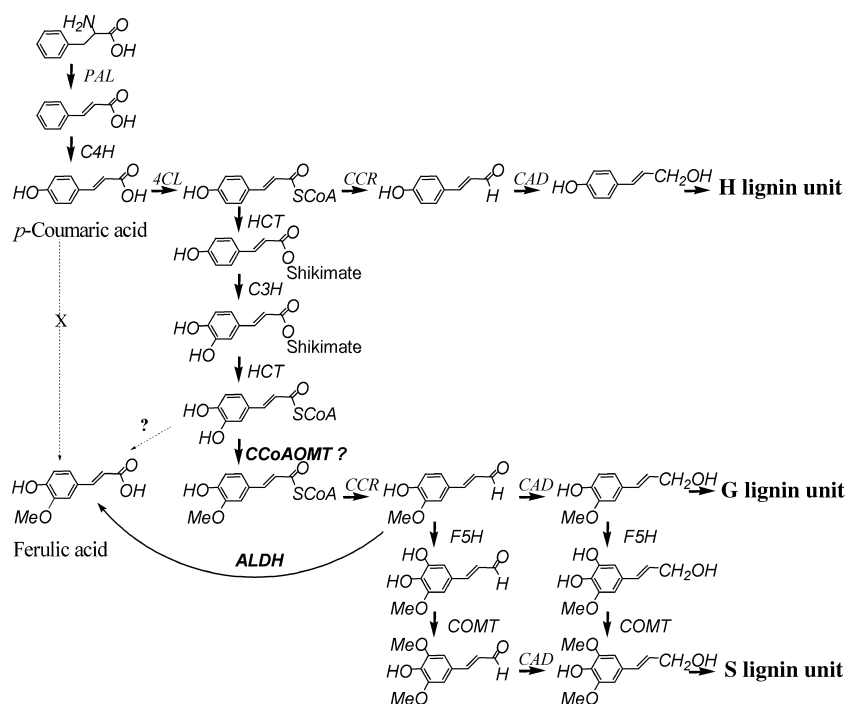


Figure 1. Current pathway for the biosynthesis of monolignols and wall-bound ferulic acid (Boudet *et al.*, 2003). See text for an explanation of the enzyme abbreviations. The dotted arrow refers to recent findings that argue against the formation of ferulic acid directly from coumaric acid via caffeic acid (Nair *et al.*, 2004).

monolignol biosynthesis to methylate 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol (Figure 1).

Lignin has a negative impact on forage digestibility (Hatfield *et al.*, 1999; Jung and Vogel, 1986), and modifying the lignin content and composition can improve the digestibility of the forage legume alfalfa (*Medicago sativa* L.) to ruminant animals (Baucher *et al.*, 1999; Guo *et al.*, 2001; Reddy *et al.*, 2005). These, and related studies addressing the impact of lignin modification on the paper pulping of trees (Halpin and Boerjan, 2003), have targeted various enzymes of the monolignol pathway for downregulation, primarily using the antisense strategy.

In spite of an increasing literature on the genetic modification of lignin content and composition in plants (reviewed in Anterola and Lewis, 2002; Boerjan *et al.*, 2003), there is as yet no systematic study in which each enzyme of the putative monolignol pathway has been downregulated in the same species and genetic background. Generation of such a series of transgenic plants would facilitate evaluation of the enzymatic controls determining both the content and composition of lignin, free from complications associated with either species differences or additional genetic factors. We here report the biochemical analysis of transgenic alfalfa lines independently downregulated in seven enzymes of the monolignol pathway, using a phenylpropanoid pathway-specific promoter in contrast to the constitutive promoters used in most previous studies on lignin engineering (Anterola and Lewis, 2002). Our results generally support the current monolignol biosynthetic pathway, but question the role of

CCoAOMT in the biosynthesis of syringyl lignin and wall-bound ferulate in alfalfa.

Results

Transgenic alfalfa lines downregulated in each of seven enzymes in the monolignol pathway

Transgenic alfalfa lines downregulated in the three cytochrome P450 enzymes of the lignin pathway (cinnamate 4-hydroxylase, C4H; 'coumarate 3-hydroxylase', C3H; 'ferulate 5-hydroxylase', F5H) (Figure 1) (Reddy *et al.*, 2005), and in the two *O*-methyltransferases (COMT and CCoAOMT) (Guo *et al.*, 2000), have been described previously. Additional lines downregulated in COMT and CCoAOMT were generated using RNAi constructs, as described in Experimental procedures. Antisense constructs for the downregulation of L-phenylalanine ammonia-lyase (PAL) and hydroxycinnamoyl CoA: quinate/shikimate hydroxycinnamoyl transferase (HCT) used either a coding region of *M. sativa* PAL or the full-length *Medicago truncatula* HCT open reading frame. All constructs were driven by the bean PAL2 promoter, which exhibits strong expression in alfalfa vascular tissue (Guo *et al.*, 2001).

Effects of the antisense C4H, C3H, F5H, and the original COMT and CCoAOMT transgenes on target transcripts and enzymatic activities in stem tissues have been presented elsewhere (Guo *et al.*, 2000; Reddy *et al.*, 2005). RNA gel blot analysis of selected PAL and HCT transformants is presented in Figures 2a, b. In each case, two lines had significantly

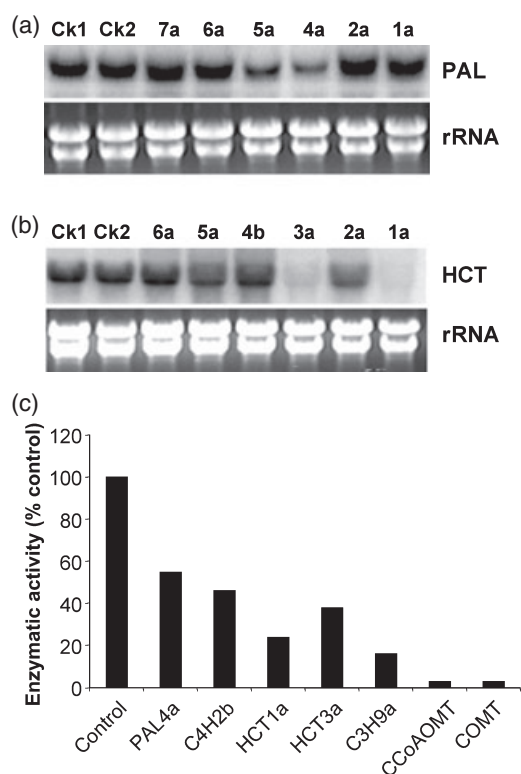


Figure 2. Downregulation of target monolignol pathway enzymes in transgenic alfalfa.

(a) RNA gel blot analysis of total RNA from stems (internodes 2–5) from two independent controls (Ck) and six independent lines expressing an antisense L-phenylalanine ammonia-lyase (PAL) construct. rRNA shows ribosomal RNA bands on the formaldehyde agarose gel before transfer as a loading control. (b) As above, showing hydroxycinnamoyl transferase (HCT) transgenics. (c) Target enzyme activities in the stems of control and alfalfa plants expressing antisense constructs for the downregulation of PAL, C4H, HCT, C3H, CCoAOMT or COMT. Activities (mean of two measurements per line) for PAL and HCT were determined in the present work. Data for other lines are from (Guo *et al.*, 2001; Reddy *et al.*, 2005). The COMT and CCoAOMT lines were AC310 and ACC305, respectively (Guo *et al.*, 2001). Values are given as a percentage of wild-type enzyme activity.

reduced target transcript levels. Figure 2c summarizes the target enzyme activities for selected C4H, C3H, COMT and CCoAOMT downregulated lines (Guo *et al.*, 2000; Reddy *et al.*, 2005), along with either the PAL or HCT activities of the most downregulated lines represented in Figure 2a,b.

Collectively, the above transgenic plants provide material for analyzing the impact of downregulation of every known enzyme of the monolignol pathway, except for 4-coumarate CoA ligase (4CL), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), in the same genetic background.

Multi-site modulation of the monolignol pathway has both predicted and paradoxical effects on lignin composition

For each targeted lignin pathway gene, either two or more independent transgenic lines were selected for strong anti-

sense downregulation by RNA gel blot and/or enzyme activity analysis. Selected lines and empty vector controls were grown in parallel in the greenhouse. Targeting PAL, C4H, HCT or C3H resulted in a greater decrease in AcBr lignin content than did the downregulation of the later enzymes (CCoAOMT, F5H or COMT) (Figure 3a), even though the extents of enzyme activity downregulation varied for different targets (Figure 2c). HCT downregulated plants showed the greatest reduction in lignin content (approximately 50% reduction of AcBr lignin and 75% reduction of total thioacidolysis monomer yield).

Downregulating each monolignol pathway enzyme resulted in an apparent reduction of the total thioacidolysis yield per gram of lignin (Figure S1a). This may reflect an increased proportion of condensed lignin units (carbon–carbon bond linked units) (Lapierre *et al.*, 1995). The trend of thioacidolysis yield data on the basis of extractive-free cell wall residues was similar to the AcBr lignin results for the corresponding lines, with the exception that COMT down-

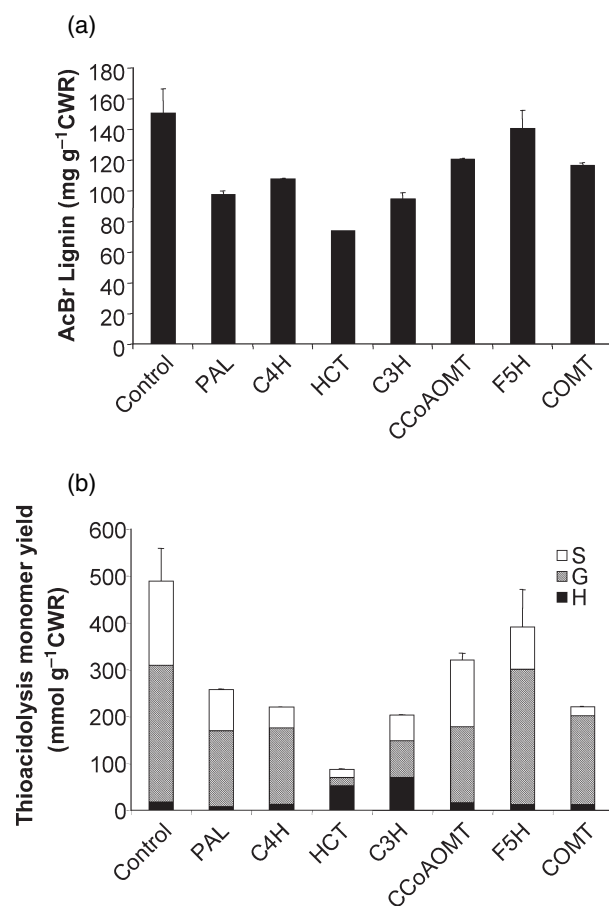


Figure 3. Lignin content in the internodes 1–8 of either control or transgenic alfalfa plants downregulated in expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT ($n = 2$).

(a) AcBr lignin levels.

(b) Thioacidolysis yields for H, G and S monomers.

regulation gave a greater reduction in thioacidolysis yield than in AcBr lignin level (compare Figures 3a and b).

There was a near linear relationship between AcBr lignin content and total thioacidolysis monomer yield based on the average values from the seven sets of downregulated lines (Figure S1b). The value of the intercept of the regression line (5.5% cell wall residue [CWR]) may represent potential contaminating material such as aromatics, proteins, carbohydrates or condensed *p*-hydroxyphenyl (H) lignin deposited during earlier stages of development (Patten *et al.*, 2005). When the total thioacidolysis monomer yields were corrected for this, most of the transgenic lines, including the H lignin-rich C3H and HCT downregulated plants, gave similar total thioacidolysis monomer yields per gram of AcBr lignin (Figure S1c). As with the unadjusted data, the exception was the COMT downregulated lines.

There were striking differences in the lignin composition dependent upon the targeted gene (Figures 3b, 4), but independent of the degree of reduction of total lignin content. Downregulation of either C3H or HCT gave a massive increase in the proportion of H units in the lignin, consistent with the position of these enzymes in the pathway prior to the insertion of the second (3-position) hydroxyl group. Reducing either F5H or COMT activity significantly reduced the number of S units, resulting in decreased S/G ratios, as previously described (Guo *et al.*, 2000; Reddy *et al.*, 2005), and is consistent with the position of these enzymes in the pathway (Figure 1). PAL downregulation reduced the overall flux into the pathway, resulting in reduced lignin content (Figure 3a) with little effect on monomer composition (Figure 4).

The lignin compositions of plants with either reduced C4H or CCoAOMT activities were not consistent with the positions of these two enzymes in the pathway. Downregulating C4H should parallel the reduction of PAL activity, as both

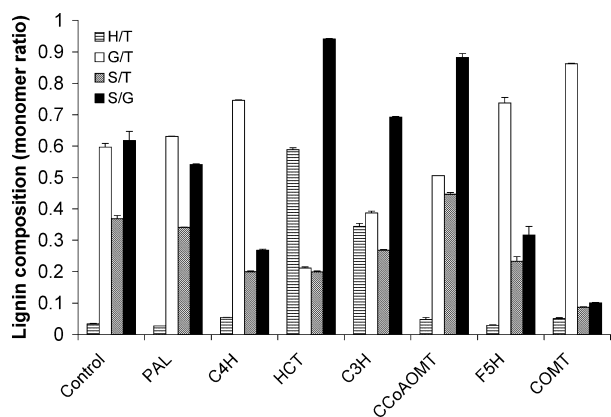


Figure 4. Lignin monomer compositions (ratios) in the internodes 1–8 of either control or transgenic alfalfa plants downregulated in expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT ($n = 2$). Ratios are for individual monomers compared with either one another (e.g. S/G) or with the total monomer yield (e.g. S/T).

enzymes function prior to the ring substitution reactions. However, C4H downregulation reduced the S/G ratio, as seen in the F5H transgenics, whereas S/G ratios were unchanged in PAL lines with a similar level of reduction in PAL enzyme activity. The position of CCoAOMT in the pathway would suggest that its downregulation should reduce both S and G units, as reported previously in tobacco and poplar (Meyermans *et al.*, 2000; Zhong *et al.*, 1998). However, CCoAOMT antisense lines with a severe reduction in enzyme activity had reduced G lignin levels but wild-type S lignin levels (Figure 3), resulting in an increased S/G ratio (Figure 4) consistent with the previous analysis of these lines (Guo *et al.*, 2000).

Multi-site downregulation of lignin biosynthesis does not disturb developmental patterns of lignification

Measurement at a single developmental stage may not resolve whether observed changes reflect either true metabolic results of lignin pathway modification or changes in developmental programs (e.g. either accelerated or delayed lignification) (Anterola and Lewis, 2002). Lines downregulated in either C4H or C3H exhibit a distinct reduction in growth rate (Reddy *et al.*, 2005), and this was also observed in the present work for the PAL and HCT lines. We therefore compared lignin content and composition at various developmental stages. Mature stems comprising eight internodes were harvested from each line and separated into individual internodes with internodes 1–2 representing the earliest developmental stage. Lignin content was reduced in all the transgenic plants (except for the F5H line) at all developmental stages (Figure 5, Table S1). All the lines showed the dominant deposition of H and G lignin at early stages of development, with increasing deposition of S lignin at later stages (Figure 5b). The H lignin levels and H/T ratio continued to increase with increasing maturity in stems of C3H and HCT downregulated lines, although in the latter case the thioacidolysis yield was lower in internodes 6–8 than in internodes 4 and 5 (Figure 5b, Table S1). The increase in the S/T ratio characteristic of development in alfalfa stems was observed in all lines except those downregulated in COMT, and was significantly reduced in the F5H and C4H downregulated lines. Parallel with the increase in the S/G ratio, the G/T ratio decreased somewhat in all lines, but to a greater extent in the CCoAOMT, PAL, C3H and HCT downregulated lines (Table S1). Overall, these data suggest that the effects of enzyme downregulation on lignin content and composition are the direct result of altered flux into the monolignol pathway, rather than altered developmental patterns.

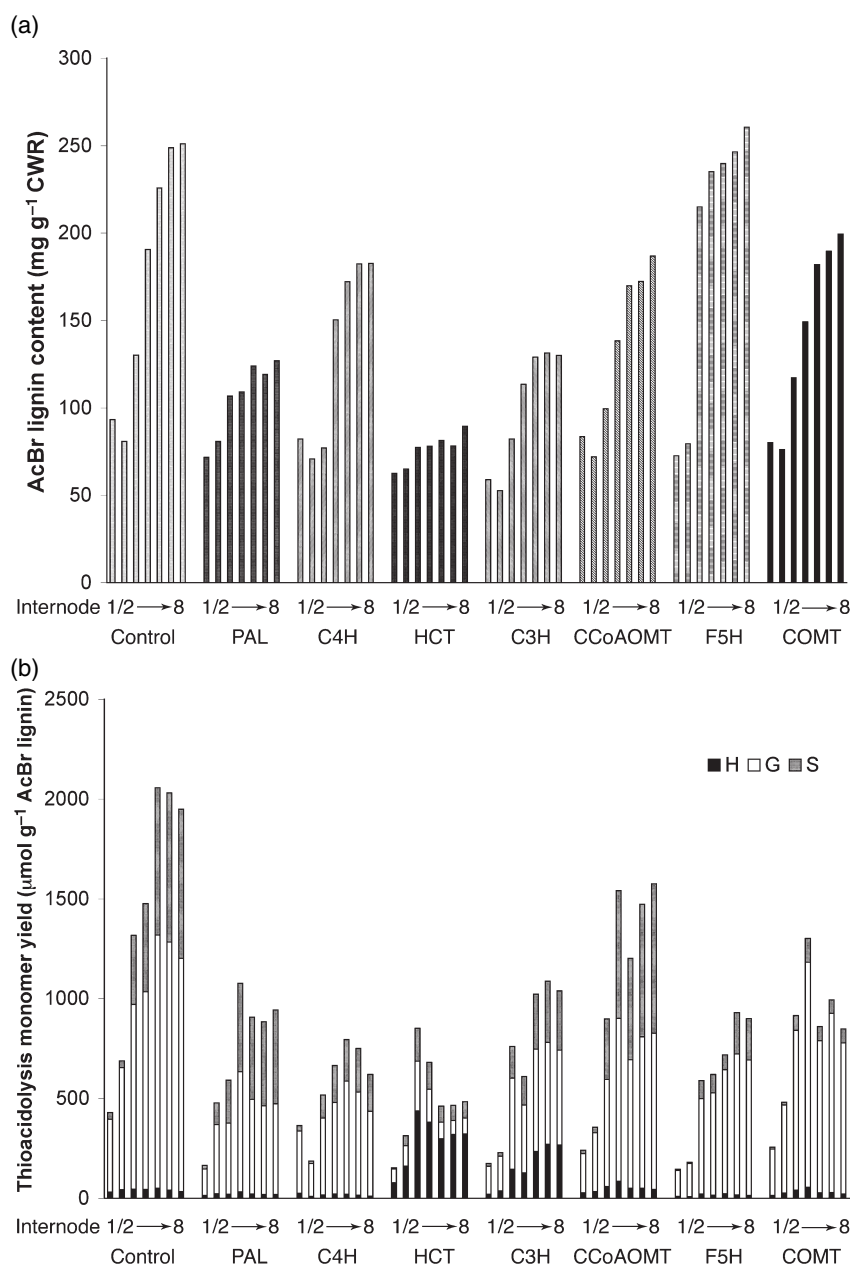
CCoAOMT is not necessary for S lignin formation in alfalfa

To verify the paradoxical lack of effect of CCoAOMT downregulation on S lignin, we generated 9–10 additional inde-

Figure 5. Lignin content and composition in individual internodes (1–8) of stems from either control or transgenic alfalfa plants downregulated in expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT (mean of two measurements except for internodes 1 and 2 where one measurement was made because of the limited sample number).

(a) AcBr lignin content of the total cell wall residue (overall coefficient of variation less than 5.6%).

(b) Thioacidolysis yield of individual monomers as a function of AcBr lignin (overall coefficient of variation less than 6.2%).



pendent lines in which CCoAOMT and COMT activities were downregulated by RNAi (Wesley *et al.*, 2001) (Figure S2a,b). Total thioacidolysis yield from stem material was reduced in all nine CCoAOMT and eight COMT lines with reduced enzyme activity (Figure 6a), but much more so in the COMT lines. This likely reflects the resistance to thioacidolysis of the linkages formed between the 5-hydroxyguaiacyl units characteristic of lignin from COMT downregulated plants (Ralph *et al.*, 2006). AcBr lignin was reduced by 15–20% in these plants (data not shown). Strikingly, for eight out of the nine CCoAOMT RNAi lines, the thioacidolysis yield of S units was essentially the same as that observed in the control lines. Equally striking was the differential effect on the S/G

ratio of either CCoAOMT or COMT downregulation (Figure 6b); CCoAOMT downregulation resulted in increased S/G ratio in all nine lines examined, whereas the characteristic strong reduction in S/G ratio (Guo *et al.*, 2000; Marita *et al.*, 2003) was observed in all plants in which COMT activity was strongly reduced.

The above results prompted us to re-evaluate a previous report of reductions in both S and G lignin in tobacco with reduced CCoAOMT activity. The CCoAOMT downregulated tobacco material (Zhong *et al.*, 1998) was kindly provided by Dr Zheng-Hua Ye (University of Georgia, Athens, GA, USA). Re-analysis of this material by our methods confirmed an approximately 50% reduction of AcBr lignin

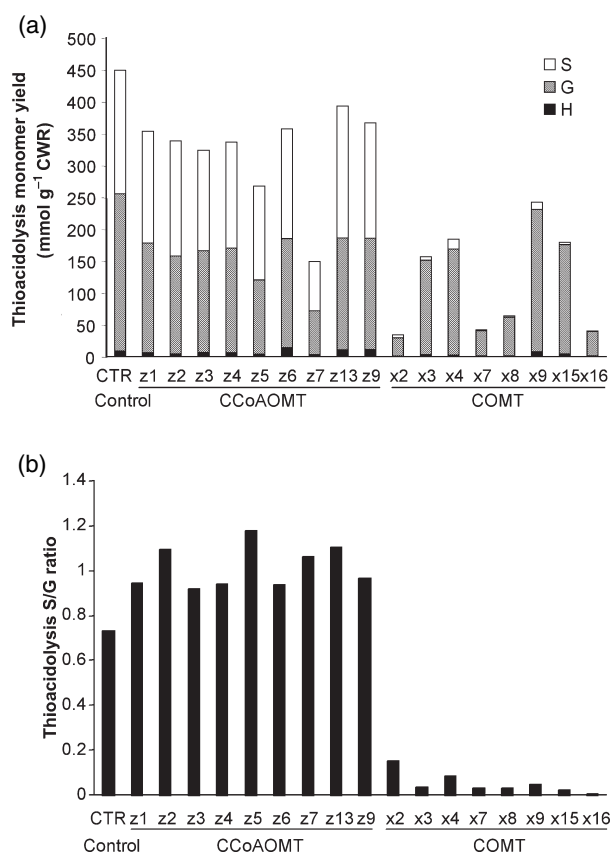


Figure 6. Lignin content and composition of individual transgenic alfalfa lines downregulated in expression of either CCoAOMT or COMT by RNAi. (a) Thioacidolysis yields of individual monomers from lignin from stems (internodes 2–5) of CCoAOMT and COMT downregulated lines. (b) S/G ratios for the above lines.

content, with a greater reduction of S units than G units (data not shown).

CCoAOMT is not necessary for wall-bound ferulate biosynthesis in alfalfa

Plant cell walls contain hydroxycinnamic acids esterified to polysaccharides. These may serve as 'anchor sites' for lignin deposition (Grabber *et al.*, 1995, 2000), and are readily removed from isolated walls by alkaline hydrolysis (Grabber *et al.*, 1995).

HPLC analysis of alkali-released wall-bound compounds from empty vector control alfalfa plants revealed the presence of ferulic and *p*-coumaric acids, *p*-hydroxybenzaldehyde and vanillin. As would be expected by their positions in the monolignol pathway after the formation of coniferaldehyde, the downregulation of either F5H or COMT did not reduce the levels of wall-bound phenolic compounds (Figure 7a). In contrast, the reduction of C3H activity resulted in decreased ferulic acid and vanillin levels, with corresponding increases in *p*-coumaric acid and *p*-hydroxybenzaldehyde, consistent

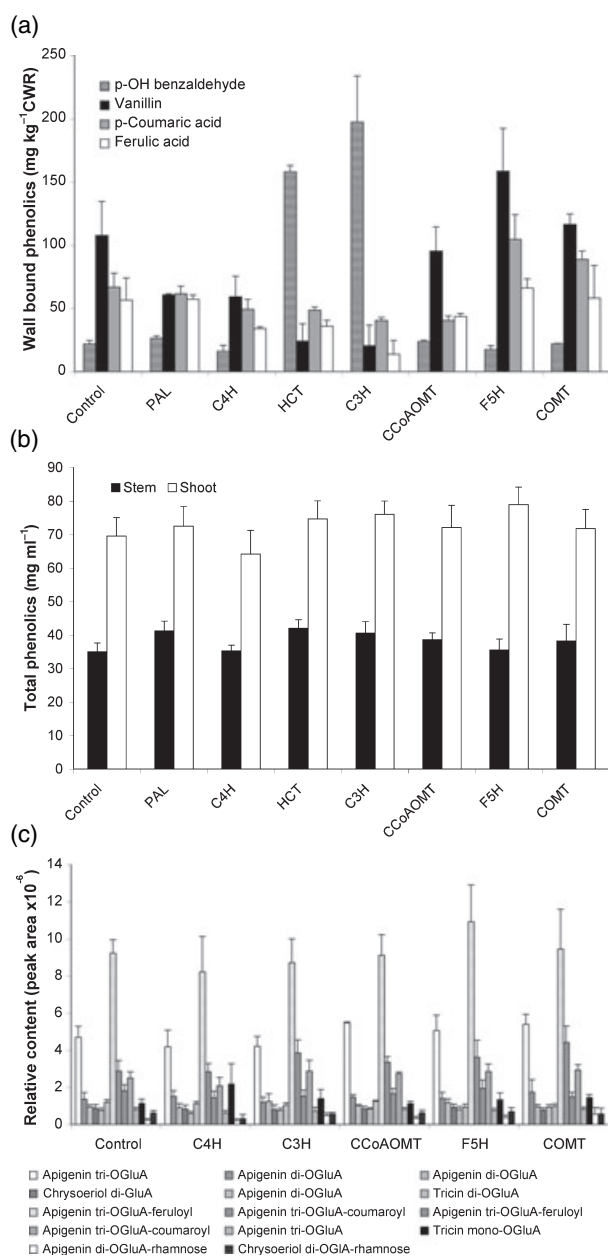


Figure 7. Wall-bound and soluble phenolic compounds in control and transgenic alfalfa plants downregulated in expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT.

(a) Compounds released by alkaline hydrolysis of cell wall material from stem internodes 1–8 ($n = 4$). (b) Total phenolic compounds in methanolic extracts from the internodes 1–8 of either stems or shoots (stems plus leaves) ($n = 2$). (c) Flavone glucosides in stems plus leaves (internodes 1–8) of control and transgenic plants downregulated in C4H, C3H, CCoAOMT, F5H and COMT ($n = 5$).

with reduced flux through the 3-hydroxylation reaction. Predictably, the downregulation of HCT mirrored that of C3H in producing a striking increase in wall-bound *p*-hydroxybenzaldehyde (biosynthetically 'equivalent' to the H units found in the lignin of HCT and C3H transgenics), and levels

of vanillin and ferulic acids were reduced in the PAL and C3H transgenics, consistent with overall reduced flux into the pathway. Surprisingly, downregulation of CCoAOMT had no statistically significant effect on levels of wall-bound ferulic acid and vanillin in either the two antisense lines or the RNAi lines (Figure S3).

Multi-site modulation of monolignol biosynthesis does not affect levels of soluble phenolic compounds

Blocking an enzymatic step in a complex pathway may result in the accumulation of either intermediates or metabolites thereof, the identification of which might help address the position of the enzyme in the pathway. There were no significant differences in levels of total phenolic compounds, determined using the Folin–Ciocalteu method (Singleton *et al.*, 1999), in either shoots (stems plus leaves) or stems alone following the downregulation of any of the seven enzymes (Figure 7b). Furthermore, bean PAL2 promoter-mediated downregulation of either F5H or COMT had little qualitative or quantitative effect on HPLC profiles of soluble phenolics (Figure S4). In contrast, new compounds appeared in extracts from stems of C4H, C3H and CCoAOMT downregulated plants (Figure S4a). Most of the compounds present in stems occurred at higher levels in shoot extracts (Figure S4b), and the major peaks were identified by LC/MS as glucuronides of the flavonoids apigenin, luteolin, chrysoeriol and tricetin, several of which were acylated with either ferulic or coumaric acids (Table S2) (Stochmal *et al.*, 2001a,b, 2001c). Downregulation of the targeted monolignol pathway enzymes did not dramatically alter the levels of these flavonoids (Figure 7c). In particular, neither downregulation of C4H nor C3H appeared to affect *p*-coumaric and ferulic acid availability for the formation of apigenin coumaroyl glucuronide and apigenin feruloyl glucuronide.

Discussion

Predicted and paradoxical effects of downregulation of lignin pathway genes on lignin content and composition

By independently downregulating multiple different genes in the monolignol pathway, a set of transgenic plants was obtained with a wide range of different lignin profiles (content and composition). Independently downregulating each enzyme decreased the overall carbon flux into lignin, and the downregulation of earlier enzymes in the pathway resulted in a more dramatic decrease in lignin content than did the downregulation of later enzymes, irrespective of the degree of enzyme activity reduction.

Severely downregulating either F5H or COMT blocks the synthesis of sinapyl alcohol, but may also increase flux into either coniferyl alcohol (Piquemal *et al.*, 2002) or, in the case of COMT, 5-hydroxyconiferyl alcohol, as suggested by the

only moderate decrease of lignin content (Guo *et al.*, 2000; this work) and the presence of 5-OH guaiacyl units in the lignin (Guo *et al.*, 2000; Marita *et al.*, 2003). Coniferyl and sinapyl alcohols can cross-couple with 5-hydroxyconiferyl alcohol to give extended chains of benzodioxane units (Marita *et al.*, 2003; Morreel *et al.*, 2004). The benzodioxane ring remains intact during lignin compositional analysis by the derivatization followed by reductive cleavage (DFRC) method, and is most likely to remain intact during thioacidolysis, so no 5-hydroxyguaiacyl monomers are released (Guo *et al.*, 2001; Marita *et al.*, 2003). There is currently no reliable method available to quantitatively measure the 5-hydroxyguaiacyl units in lignin (Marita *et al.*, 2003).

Coniferyl alcohol and small quantities of *p*-coumaryl alcohol are co-polymerized into the primary wall at early stages of lignification; coniferyl alcohol and increasing quantities of sinapyl alcohol are then deposited to form mixed G and S lignins during secondary wall development (Grabber, 2005; He and Terashima, 1990, 1991). In the present study lignin content increased with stem maturity, irrespective of the enzyme targeted for downregulation. The early lignins were mostly of the G-rich type (80–90% molar ratio) with a relatively high number of H units (5–10%). With increased maturity, S units were deposited and the relative number of H units decreased in all but the C3H and HCT downregulated lines, in which they continued to increase to 25% and 66%, respectively. This continued accumulation of H lignin in these lines indicates that *p*-coumaryl alcohol is not only incorporated into primary walls at early stages of lignification, but also partially substitutes for coniferyl and sinapyl alcohols in secondary walls at later development stages. The coupling and cross-coupling reactions of *p*-coumaryl alcohol in C3H downregulated plants are analogous to those of coniferyl and sinapyl alcohols (Ralph *et al.*, 2006).

HCT and C3H are together responsible for the 3-hydroxylation in the conversion of coumaroyl CoA to caffeoyl CoA (Figure 1). However, whereas downregulation of C3H had no effect on S/G ratio, HCT downregulation strongly increased S/G, in contrast to the relative reduction in S units caused by silencing HCT in tobacco (Hoffmann *et al.*, 2004). A similar unexpected increase in the S/G ratio was seen in C4H downregulated alfalfa (this work) and tobacco (Sewalt *et al.*, 1997). This is paradoxical in view of PAL downregulation either causing no effect on the S/G ratio (this work) or increasing the S/G ratio in tobacco (Sewalt *et al.*, 1997). Simplistically, downregulating either PAL or C4H should reduce the overall flux into the monolignol pathway, with equivalent effects on lignin content and composition.

Measurement of either transcript levels or enzymatic activity *in vitro* only provides an estimate of the extent of downregulation of a particular biosynthetic step *in vivo*. However, on the basis of determination of total lignin levels, C4H and PAL downregulation are likely to have had similar

impacts on the overall pathway flux. This suggests that the differences in lignin composition in the PAL and C4H transgenic lines is not simply the result of different overall fluxes into the pathway determined by the extent of individual enzyme downregulation.

Metabolic channeling may operate at several steps in phenylpropanoid biosynthesis (Winkel, 2004). For example, one of the two tobacco PAL isoforms appears to exist in a membrane-bound complex with C4H (Achnine *et al.*, 2004). Differential association of alfalfa PAL isoforms with C4H might explain the different effects on lignin composition of downregulating either PAL or C4H, although there is as yet no direct evidence for separate metabolic channels leading to G and S lignin, and no evidence was obtained for channeling between alfalfa COMT and F5H (Guo *et al.*, 2002).

The opposite effects on S/G ratios in plants downregulated in genes at similar points in the monolignol pathway might also reflect changes in lignin structure (i.e. linkage pattern) that are known to affect the thioacidolysis yields of the different monomeric units (Lapierre *et al.*, 1995).

Downregulation of early lignin pathway genes affects wall-bound ferulate levels

Mild alkali hydrolysis of cell walls released *p*-hydroxybenzaldehyde, vanillin, *p*-coumaric acid and ferulic acid. *p*-Hydroxybenzaldehyde and vanillin may be produced from either wall-bound *p*-hydroxycinnamate or lignin terminal groups under the hydrolysis conditions used here (Sun *et al.*, 2001). Consistent with the pathway in Figure 1, downregulation of either F5H or COMT did not affect wall-bound hydroxycinnamates, whereas PAL and C4H downregulated lines produced less wall-bound phenolics. The downregulation of C3H and HCT resulted in a dramatic decrease of *m*-methoxylated hydroxycinnamates (vanillin plus ferulic acid) with a corresponding 8–10-fold increase in non-substituted *p*-hydroxycinnamates (*p*-hydroxybenzaldehyde plus *p*-coumaric acid), confirming that cell wall ferulate shares a common biosynthetic pathway with the monolignols through the C3H and HCT reactions in alfalfa.

Downregulation of lignin pathway genes does not affect flavonoid synthesis

Using the bean PAL2 promoter (Guo *et al.*, 2001; Liang *et al.*, 1989) to drive the downregulation of lignin/phenylpropanoid pathway genes does not significantly change the levels of acylated flavonoids in alfalfa. PAL and C4H are early steps in the formation of 4-coumaroyl CoA, the key intermediate in flavonoid synthesis, and their constitutive 35S promoter driven downregulation has major impacts on the levels of soluble and wall-bound phenylpropanoid compounds in tobacco (Blount *et al.*, 2000; Korth *et al.*, 2001). The PAL2

promoter is expressed strongly in both the vascular and epidermal tissue of transgenic alfalfa (Guo *et al.*, 2001). The lack of effect of phenylpropanoid pathway gene downregulation on coumaroylated and feruloylated flavonoids suggests that the phenylpropanoid scaffolds for the flavonoid B-ring and acyl substituents are not produced in the same cell types that synthesize lignin precursors.

CCoAOMT downregulation causes accumulation of hydroxycinnamic acid 3-*O*-glucosides in poplar (Meyermans *et al.*, 2000) and alfalfa (Chen *et al.*, 2003; Guo *et al.*, 2001), and COMT downregulation results in the accumulation of compounds derived from either 5-hydroxyconiferyl alcohol or 5-hydroxyconiferinaldehyde in the xylem of poplar (Morreel *et al.*, 2004). Caffeic acid 3-*O*-glucoside and other novel phenolics were detected in stem extracts from CCoAOMT, C3H or C4H downregulated alfalfa, but the overall concentrations of soluble phenolics in stems plus leaves were similar in control and lignin-modified plants. Principal component analysis of HPLC chromatograms could not resolve differences in leaf metabolite profiles between wild-type and either COMT or CCoAOMT downregulated transgenic plants of the same genetic background (Chen *et al.*, 2003). Similarly, the downregulation of HCT by virus-induced gene silencing in tobacco resulted in no significant change in the levels of soluble leaf phenolics (Hoffmann *et al.*, 2004). A C3H T-DNA insertion mutant of *Arabidopsis* with lignin essentially made of H units had elevated levels of flavonol glycosides, but very low levels of sinapate esters (Abdulrazzak *et al.*, 2006). However, in *Arabidopsis* plants in which C3H was downregulated by co-suppression, the levels of *p*-coumaroyl malate and sinapoyl malate were not affected, and sinapoylated cyanidin glucosides accumulated (Abdulrazzak *et al.*, 2006). Our results indicating a lack of effect of C3H downregulation on levels of feruloylated flavonoids likewise support the hypothesis of an alternative pathway for the 3-hydroxylation of the acyl group of acylated flavonoids.

The role of CCoAOMT in monolignol and ferulate biosynthesis

The most striking result of the present study was the lack of effect of CCoAOMT downregulation on the accumulation of either S lignin or wall-bound ferulate in ten independent transgenic alfalfa lines. The only exception was RNAi line Z7, in which both G and S lignin were strongly downregulated, although the overall S/G ratio was elevated as in all other CCoAOMT lines. CCoAOMT activity was higher in line Z7 than in several of the other RNAi lines, or the antisense line. The extreme lignin reduction in line Z7 may be a secondary effect owing to the transgene insertion site, or a somaclonal phenomenon.

Overall, our results suggest that the pathway in Figure 1 is questionable, and that the S lignin pathway may operate

independently of G lignin biosynthesis in alfalfa. It was recently proposed that ferulate is synthesized from the lignin pathway intermediate coniferaldehyde via the action of an aldehyde dehydrogenase enzyme (Nair *et al.*, 2004) (Figure 1). A model that excludes the G-lignin pathway CCoAOMT from introducing the 3-methoxyl function in S lignin (and ferulate) provides additional flexibility for the cellular control of lignin composition.

Either COMT (acting at the level of free caffeic acid) or an alternate OMT, acting at the level of an as yet unidentified ester, could introduce the 3-methoxyl group into S monolignol, and ferulic acid, precursors. Because C3H and HCT downregulation affect wall-bound ferulate levels, the OMT clearly operates at a stage subsequent to the formation of caffeoyl shikimate. Free caffeic acid could be formed by either hydrolysis or trans-esterification of caffeoyl CoA.

The model legume *M. truncatula* (May and Dixon, 2004) contains multiple genes annotated as encoding either COMT- or CCoAOMT-like enzymes (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago), which may encode either isozymes or related OMTs with different substrate preferences. Several of these, in addition to the functionally identified COMT and CCoAOMT genes targeted in the present work, are expressed in stem tissue (C. Barnwell, M.S.S. Reddy and R.A. Dixon, unpublished results), and are therefore candidates for involvement in monolignol biosynthesis. Studies are in progress to further elucidate their tissue-specific expression patterns and *in vitro* substrate preferences in order to determine if they might function in monolignol and ferulate biosynthesis.

Experimental procedures

Plant material and sample collection

Alfalfa (*M. sativa* cv Regen SY) plants were grown in the greenhouse with a 16-hour day facilitated by supplementary lighting. Genetic transformations were performed with clonally propagated material of one highly regenerable line. A 1834-bp part of the coding region of alfalfa PAL (GenBank accession X58180) was amplified by PCR using forward (ATGGAACAATATCAGCAGCTATCACA) and reverse (CACCTAGTGCATGATCTACCAACTTGCCTTAG) primers. Similarly, the entire coding region of *M. truncatula* HCT (TC106825; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago) was amplified using forward (ATGATCATAAACGTTAGAGATTTCG-ACAATG) and reverse (CACCTCAAATATCATAAGAAGTCCTTG-AACAC) primers. pCAMBIA2200-MsPALas and pCAMBIA2200-MtHCTas constructs were made for antisense-mediated downregulation of PAL and HCT respectively, as described previously (Reddy *et al.*, 2005). The generation of transgenic alfalfa lines was carried out as reported previously (Guo *et al.*, 2001; Reddy *et al.*, 2005). A 300-bp region of the alfalfa COMT and CCoAOMT coding sequences was taken to make alfalfa COMT and CCoAOMT RNAi constructs (Wesley *et al.*, 2001), which were transformed into the alfalfa line R2336 (Forage Genetics International, West Salem, WI, USA) using the procedure of Samac and Temple (2004). COMT and CCoAOMT RNAi lines were characterized by enzyme activity measurements as

described previously (Guo *et al.*, 2001). Plant material was collected when stems had produced eight internodes. Leaves and stems were separated, frozen in liquid nitrogen, ground into a powder with a mortar and pestle under liquid nitrogen, and freeze dried.

RNA gel blot analysis

Total RNA was isolated from 500 mg of ground stem tissue (internodes 2–5) using TRIAGENT solution (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA (10 µg) was separated on 1.2% formaldehyde-containing agarose gels and transferred onto a nylon membrane (Hybond-N+; Amersham Biosciences Corp, Piscataway, NJ, USA) by standard procedures (Sambrook *et al.*, 1989) and UV cross-linked using a Stratalinker (Stratagene, La Jolla, CA, USA). The PAL and HCT coding sequences were labeled with a [α -³²P]dCTP labeling kit (Amersham) and purified on Probe Quant G50 micro columns (Amersham). The purified probes were used for RNA gel blot hybridization as described previously (Reddy *et al.*, 2002).

Enzyme activity assay

Alfalfa stems (internodes 1–6) were harvested and ground to powder under liquid nitrogen. One gram of tissue was weighed into 12-ml tubes on ice, and 2 ml of extraction buffer containing 50 mM Tris-HCl (pH 7.0) and 0.1% β -mercaptoethanol was added. The mixture was homogenized with a polytron for 10 sec on ice. Phenolic compounds were removed by the addition of a spatula-tip-full of polyvinylpyrrolidone, vortexing and incubating on ice for 5 min. The supernatant was separated by centrifugation at 12 000 g at 4°C for 15 min, desalted through a Sephadex G-25 column, and used for assay for PAL and HCT activity. PAL activity was determined spectrophotometrically as described previously (Blount *et al.*, 2000). HCT activity was determined using *p*-coumaroyl CoA (0.5 mM) and shikimic acid (2.5 mM) as acyl donor and acceptor, respectively (Hoffmann *et al.*, 2003). Protein concentrations were determined by the Bradford procedure (Bradford, 1976).

Extraction of soluble phenolic compounds

Freeze-dried stems or shoots (100 mg) were extracted in screw-capped Pyrex test tubes (4 ml). Chloroform/methanol (2:1 v/v) with the hydrophobic internal standards docosanol (22.0 µg ml⁻¹) and docosane (20.0 µg ml⁻¹) (3 ml) was added to the tubes and the samples extracted on a shaker overnight at room temperature (20°C). Water containing the hydrophilic internal standard ribitol (0.5 mg ml⁻¹) (0.75 ml) was then added and the samples were vortexed briefly and extracted for 4 h at room temperature. After centrifuging at 3000 g for 10 min at 4°C, the chloroform and aqueous phases were separated with a micro syringe. The aqueous fractions were used directly for soluble phenolic profiling using HPLC. Quantification was by integration of identified peak areas. Total phenols in the aqueous phases were determined using Folin and Ciocalteu's reagent with gallic acid as standard (Singleton *et al.*, 1999).

Extraction of wall-bound phenolic compounds

The residue from the extraction of stem material was washed sequentially with 50, 70 and 100% methanol and finally with 100% acetone (3 ml). After removing the acetone, the samples were dried at 37°C under a stream of N₂ and weighed. Aqueous 2 N NaOH

(1.8 ml) was added and the samples were extracted overnight at 37°C in the dark. The mixtures were acidified to pH 3 with 6 N HCL and 100 µl of internal standard (3,4-dihydroxybenzaldehyde, 0.5 mg ml⁻¹ in 50% methanol) was added. After extraction with ethyl acetate (1.6 ml × 3) and separation of the layers by centrifugation, the combined organic phases were dried under a stream of N₂ and re-suspended in 70% methanol for HPLC analysis. Compounds were identified by comparing the UV spectra and retention times with those of authentic standards, and quantified by means of standard curves with correction for recovery of external standard.

HPLC analysis

HPLC was carried out on a Beckman System Gold HPLC system consisting of a programmable solvent module 126, a System Gold 508 autosampler and a System Gold 168 diode array detector. A Waters Spherisorb ODS-2 5 µ reverse phase column (5-µm particle, 250 × 4.6 mm) was used. Solvent: A, 0.1% phosphoric acid in water; B, acetonitrile. Gradient: for soluble phenolics, 8–44 % B in 55 min; for wall-bound phenolics, 5–25% B in 50 min.

LC/MS analysis

HPLC-MS used an HP 1100 liquid chromatograph coupled with a Bruker Esquire Ion-Trap mass spectrometer equipped with an electrospray source. Separation was achieved using a Waters Spherisorb ODS-2 5 µ reverse phase column (5-µm particle, 250 × 4.6 mm). Solvent: A, 1% acetic acid; B, acetonitrile. Gradient: 5–50% B in 60 min at 0.8 ml min⁻¹. Ion charge control was set at 30 000 with a maximum acquired time of 100 ms. Mass spectra were recorded over a range of 50–2200 *m/z*. Compounds were identified by comparing the UV spectra and mass spectra with literature values (Stochmal *et al.*, 2001a,b,c).

Determination of lignin content and composition

Lignin content of stem material was determined by the acetyl bromide method using approximately 15 mg extractive-free material (Fukushima and Hatfield, 2004). A molar extinction coefficient of 17.2 (determined for lignin from wild-type alfalfa) was used for all samples. Thioacidolysis methods (Lapierre *et al.*, 1985, 1995) were used to determine lignin composition. Thioacidolysis was performed using approximately 20 mg of extractive-free samples reacted with 3 ml of 0.2 M BF₃ etherate in an 8.75:1 dioxane/ethanethiol mixture. Lignin-derived monomers were identified by gas chromatography mass spectrometry (GC/MS) and quantified by GC, as their trimethylsilyl derivatives, on a Hewlett Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60-m × 0.25-mm X0.25-µm film thickness). Mass spectra were recorded in electron impact mode (70 eV) with 60–650 *m/z* scanning range.

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Supplementary Material

The following supplementary materials are available for this article online:

Figure S1. Thioacidolysis yields of lignin monomers from alfalfa plants downregulated in expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT.

(a) Thioacidolysis yields of individual monomers as a function of AcBr lignin content for each set of transgenic lines downregulated in a specific gene.

(b) Relationship between total monomer yield and AcBR lignin content for the seven sets of transgenic lines.

(c) Total monomer yields as a function of 'corrected' AcBr lignin content.

Figure S2. COMT or CCoAOMT activities in control and transgenic plants in which the targeted OMT gene had been downregulated by RNAi.

Figure S3. Levels of wall-bound phenolic compounds in alkali-hydrolyzed cell wall extracts from control, COMT or CCoAOMT downregulated alfalfa plants. Data show the means and standard deviations from two (control), nine (COMT) and 10 (CCoAOMT) independent lines.

Figure S4. HPLC chromatograms of aqueous methanol extracts of tissues from either control alfalfa plants or plants downregulated in the expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT.

(a) Internodes 1–8 of stem tissues. Arrows show the accumulated caffeoyl 3-*O*-glucoside in the CCoAOMT downregulated line and novel unidentified phenolics in other lines.

(b) Internodes 1–8 of shoot (stem plus leaves). Peak numbers are the same in Table S2.

Table S1. AcBr lignin content, thioacidolysis yields and S/G ratios determined in individual internodes of developing alfalfa stems downregulated in the expression of PAL, C4H, HCT, C3H, CCoAOMT, F5H or COMT.

Table S2. Identification of glucosylated and acylated flavone derivatives from alfalfa shoots by LC/MS.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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