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Profiling phenolic metabolites in transgenic alfalfa modified in lignin biosynthesis

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Abstract

Soluble phenolics, wall-bound phenolics and soluble and core lignin were analyzed in transgenic alfalfa with genetically down-regulated *O*-methyltransferase genes involved in lignin biosynthesis. High performance liquid chromatography and principal component analysis were used to distinguish metabolic phenotypes of different transgenic alfalfa genotypes growing under standard greenhouse conditions. Principal component analysis of HPLC chromatograms did not resolve differences in leaf metabolite profiles between wild-type and transgenic plants of the same genetic background, although stem phenolic profiles were clearly different between wild-type and transgenic plants. However, the analytical methods clearly differentiated two non-transgenic alfalfa cultivars based on either leaf or stem profiles. Metabolic profiling provides a useful approach to monitoring the broader biochemical phenotypes of transgenic plants with altered expression of lignin pathway enzymes.

Keywords: Alfalfa; *Medicago sativa*; Leguminosae; Metabolic profiling; Phenolics; Lignin

1. Introduction

Many efforts have been made to modify lignin in order to improve cell wall digestibility for ruminants, as well as to increase the efficiency of wood pulping and papermaking processes (Whetten and Sederoff, 1991; Boudet and Grima-Pettenati, 1996; Sewalt et al., 1997; Dixon et al., 2001; Guo et al., 2001; Pilat et al., 2002). In our laboratory, we have generated transgenic alfalfa (*Medicago sativa* L.) plants characterized by down-regulation of enzymes mediating important reactions in the lignin pathway, with specific emphasis on the reactions catalyzed by caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) (Guo et al., 2000, 2001). Independent down-regulation of either enzyme results in alterations to lignin content and composition that improve forage digestibility (Guo et al., 2000, 2001). More specifically, COMT down-regulation leads to a drastic reduction in dimethylated syringyl (S) monomers in the lignin, with little effect on monomethylated guaiacyl (G) monomers, whereas down-regulation of CCoAOMT results in a reduction in G units with little effect on S units (Guo et al., 2000).

In spite of the fact that most, if not all, of the enzymes involved in monolignol biosynthesis are known and their corresponding genes are cloned (Anterola and Lewis, 2002), there is still considerable uncertainty as to the exact order of reactions leading to the biosynthesis of S and G units in vivo, and whether these pathways are linked in a metabolic grid or are independently regulated (Dixon et al., 2001; Anterola and Lewis, 2002; Humphreys and Chapple, 2002; Yamauchi et al., 2003). One approach towards addressing this problem is to down-regulate the pathway sequentially at each enzymatic step, and to analyze levels of potential intermediates or spill-over products of the pathway. On a broader scale, addressing the concept of substantial equivalence between genetically modified organisms and their wild-type progenitors (Boyes et al., 2001; Roessner et al., 2001) necessitates more in depth profiling of metabolites to determine whether changes in unexpected metabolites might occur due to transgene expression.

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HPLC is a preferred separation method for hydroxylated phenylpropanoid compounds (Graham, 1991a,b), and UV detection is also simple due to the presence of absorbing chromopohores. HPLC/UV has been frequently used to characterize transgenic plants modified in phenylpropanoid biosynthesis (Howles et al., 1996; Guo et al., 2000) or to detect activity of phenylpropanoid pathway enzymes in vitro (Chen et al., 2001; Parvathi et al., 2001). To fully characterize gene function, and to define the variability in the plant metabolome and any unintended changes caused by genetic modification, the extraction, separation and identification of a wide range of compounds in a single measurement is essential (Graham, 1991b; Fiehn et al., 2000; Fraser et al., 2000; Faghihi et al., 2001). The chemical diversity and varying abundance of plant metabolites makes it difficult, if not impossible, to employ a procedure that is applicable for all, or even the majority, of plant metabolites (Fiehn, 2002). In this study, we have developed methods for profiling the soluble phenolics, wall-bound phenolics and cell wall lignins from the same transgenic alfalfa samples to further understand the effects of single gene disruption in the monolignol branch of phenylpropanoid biosynthesis.

2. Results and discussion

2.1. Optimization of extraction procedures

An HPLC protocol was developed by Graham (1991b) to simultaneously analyze a wide range of soluble aromatic secondary metabolites. It has been shown that this application is simple, sensitive and highly reproducible (Graham, 1991b). Aqueous methanol has been successfully used to extract plant phenolic compounds in our laboratory. To remove non-polar lipids that may affect later analysis, hexane was added to the methanol during the first extraction. To minimize biological variation, three plants from each line were pooled and ground under liquid nitrogen. Six injections were conducted using the same sample. For all the peaks integrated, the area variations were below 5% (average 3.9%). For replications of different extractions from the same samples, the precision of the analysis did not change significantly, the variations for all peaks integrated being less than 10%.

To optimize the extraction procedure, one-step extraction and combined extractions were evaluated. In combined extraction, plant tissue was extracted two more times with aqueous methanol and all extracts were combined. Combination of all extractions diluted the extracts and made it necessary to concentrate the samples before subjecting to HPLC analysis; during concentration, irreversible precipitation may occur. Fig. 1 shows a comparison of HPLC profiles of one-step and combined extractions of alfalfa leaves. Using one-step extraction, all the RSD values were less than 13% with an average of 5.5%, while the combined extraction procedure gives a range of 23% with an average of 9.6%. Since no new compounds were detected in the later extractions (Fig. 1), one-step extraction was used for soluble phenolic profiling to minimize variation during extraction.

Both fresh and frozen dried tissues were evaluated. Graham (1991b) has proposed a direct extraction in microfuge tubes using fresh tissues, but this does not work well for stems. Although fresh tissue was easier to extract, it is difficult to handle on a small scale, and the reproducibility is not as good as with frozen dried samples. Because of the varying abundance of plant metabolites and the non-linear response of the diode array detector to different metabolites, it is important to control the sample concentrations subjected to HPLC analysis. To obtain the best reproducibility, freeze dried samples were used in the present study. The extraction procedure provided here is simple and highly reproducible. It not only allows examination of soluble phenolic compounds from different tissue samples, but also cleans up these samples for further analysis (i.e. wall-bound phenolics and lignin analysis). Based on the RSD values reported above, our standard greenhouse growth conditions appear sufficient for providing reproducible biological material for the analysis of phenolic profiles. The extraction and analysis procedure used here can therefore provide representative sets of phenolic fingerprints of alfalfa plant material.

2.2. Soluble phenolic profiles of transgenic alfalfa with reduced COMT and CCoAOMT activities

Having determined satisfactory extraction parameters, we incorporated these into an overall procedure to characterize soluble phenolics, wall-bound phenolics, soluble lignin and core lignin in the same tissue sample. After separating the leaf and stem, the samples were pooled and ground to powder and freeze dried. Three 50 mg samples from each plant line were extracted for soluble phenolics, and from each extraction three injections were made and metabolite levels were determined.

A large number of phenolic compounds have been reported in alfalfa (Howarth, 1988). They range from simple molecules like benzoic acid to complex flavonoids, isoflavonoids and coumestans. However, there are no reports on accumulation of most of the intermediates proposed to be involved in monolignol biosynthesis. Table 1 shows the retention times and spectral characteristics of compounds involved in lignin biosynthesis and related pathways. Fig. 2 compares soluble metabolite levels in leaf and stem material from control and COMT or CCoAOMT down-regulated alfalfa plants. Definitive assignments for all the peaks in each

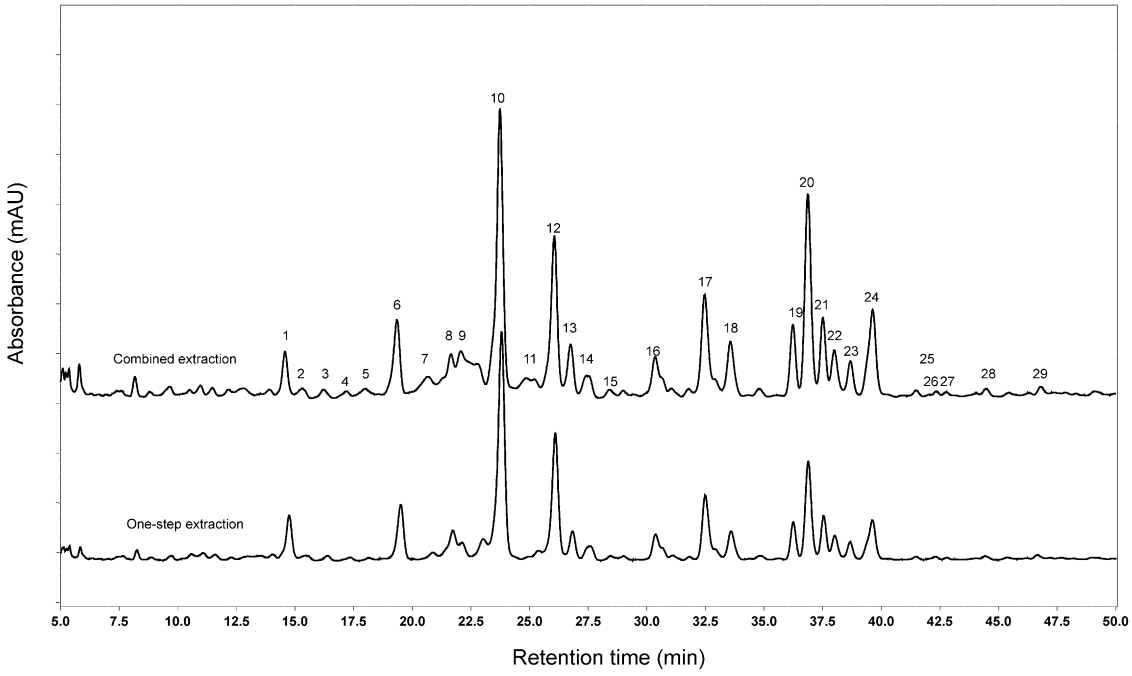


Fig. 1. HPLC profiles of one-step and combined extractions of alfalfa leaves recorded at 280 nm.

Table 1
Chromatographic behavior, spectral characteristics and detection limits for standards of monolignol precursors and related compounds (the quantifications were made at the wavelength marked with⋆)

Sample ID	Retention time (min)	Spectral characteristics (nm at λ_{max})	Limit of detection (nmol/ml)
Cinnamic acid	43.75	217, 276⋆	0.405
p-Coumaric acid	28.42	225, 309⋆	0.280
p-Coumaryl aldehyde	29.23	234, 325⋆	0.452
p-Coumaryl alcohol	18.77	210, 260⋆	0.386
Ferullic acid	29.44	218, 233, 293, 322⋆	0.417
Coniferyl aldehyde	30.46	221, 239, 302, 340⋆	0.292
Coniferyl alcohol	19.96	214, 263⋆, 299	0.488
Sinapic acid	28.93	235, 322⋆	0.361
Sinapyl aldehyde	30.49	244, 343⋆	0.216
Sinapyl alcohol	20.38	219, 306⋆	0.166
Caffeic acid	20.1	217, 238, 294, 323⋆	0.316
Caffeyl aldehyde	20.88	223, 240, 337⋆	0.317
Caffeyl alcohol	12.75	218, 262, 296⋆	0.259
5OH Ferulic acid	20.46	233, 321⋆	0.404
5OH Coniferyl aldehyde	21.97	243, 343⋆	0.407
5OH Coniferyl alcohol	39.32	224, 272⋆	0.321
3,4-Dihydroxybenzaldehyde	11.92	230, 279, 310⋆	0.290
3,4-Dihydroxy-5-methoxybenzaldehyde	13.63	219, 305⋆	0.393
Acetosyringone	22.64	222, 299⋆	0.199

chromatogram are currently being pursued via LC/MS. We are, however, particularly interested in detection of monolignol precursors and their changes in the transgenic plants. However, except for a small amount of ferulic acid, none of the metabolites listed in Table 1 could be detected in either control or OMT down-regulated transgenic plants. This indicates that the concentrations of these metabolites are very low and that down-regulation of COMT and CCoAOMT does not cause accumulation of the substrates of these enzymes.

CCoAOMT down-regulation has a greater effect on the amounts and types of soluble phenolic compounds in stems than does COMT down-regulation (Fig. 2), with the major differences being seen in the phenolic acid/ester region of the chromatogram. The phenolic acid derivative that clearly accumulates in CCoAOMT down-regulated alfalfa had previously been identified as caffeoyl glucoside (Guo et al., 2000). During monolignol biosynthesis, CCoAOMT is proposed to catalyze the methylation of caffeoyl CoA to form feruloyl CoA. It is not uncommon that, when an enzyme is down-regulated, the substrate of that enzyme may accumulate. However, caffeoyl glucoside is not a substrate for CCoAOMT (D. Guo and R.A. Dixon, unpublished results). It is also unclear as to whether caffeic acid itself is an intermediate in monolignol biosynthesis (Li et al., 2000; Parvathi et al., 2001; Franke et al., 2002). When CCoAOMT is down-regulated, caffeic acid itself may accumulate, or caffeoyl CoA levels may transiently rise and lead to formation of caffeic acid via thioesterase activity. Caffeic acid may then be glycosylated, perhaps via an endogenous detoxification mechanism (Lim et al., 2001; von Rad et al., 2001).

Compared with the corresponding profiles for stem tissue, there appears to be little impact of down-regulation

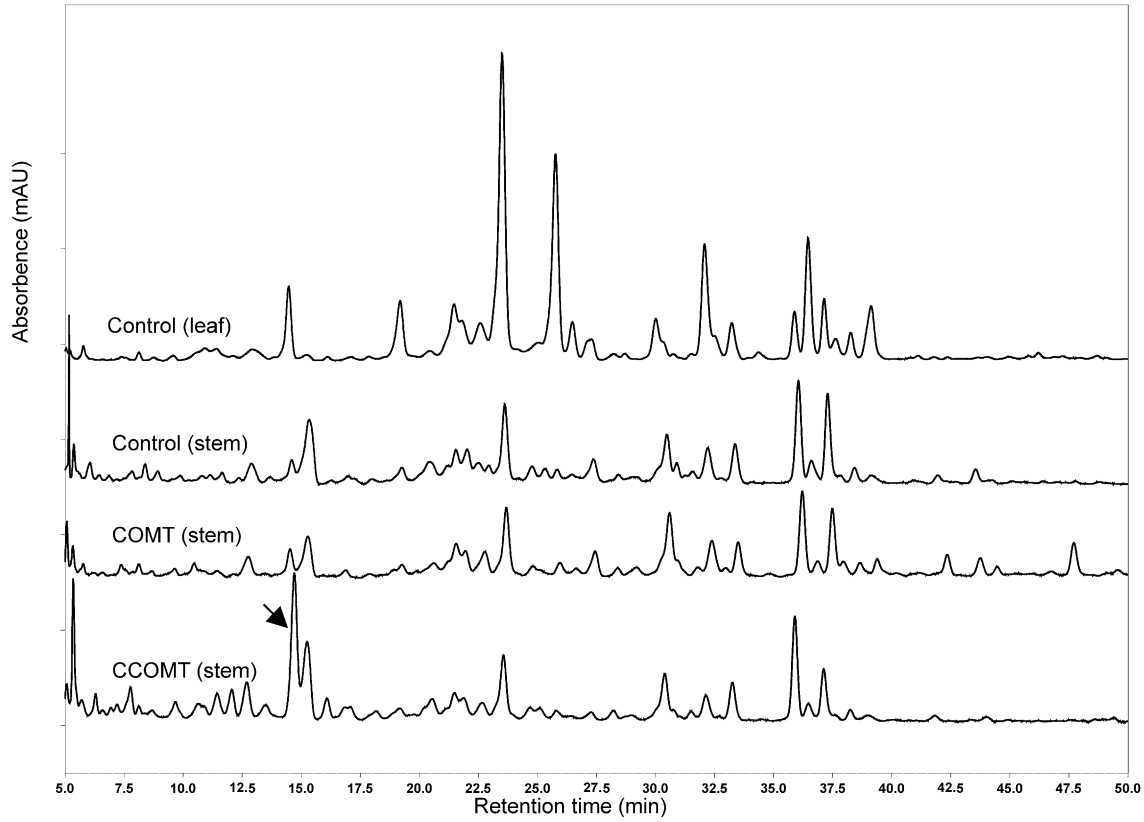
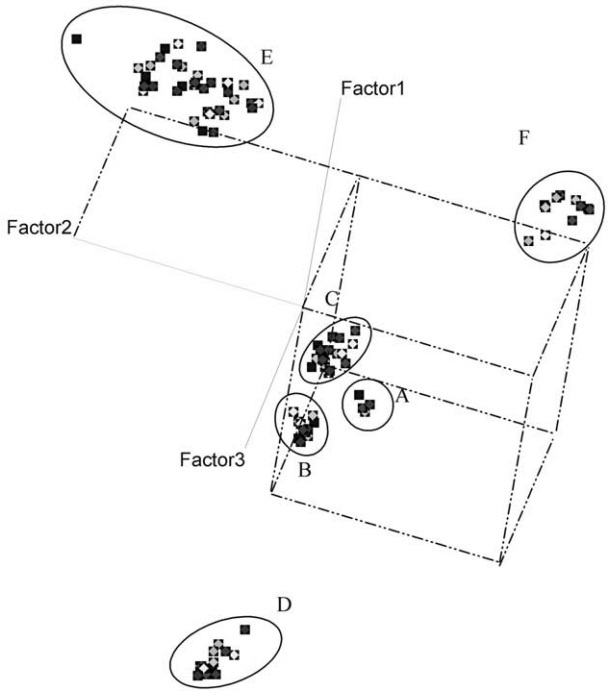


Fig. 2. Comparison of HPLC fingerprints of leaf extracts from wild-type and stem extracts from wild-type and COMT or CCoAOMT down-regulated alfalfa plants recorded at 280 nm. The arrow indicates the caffeoyl glucoside that accumulates in CCoAOMT down-regulated alfalfa.

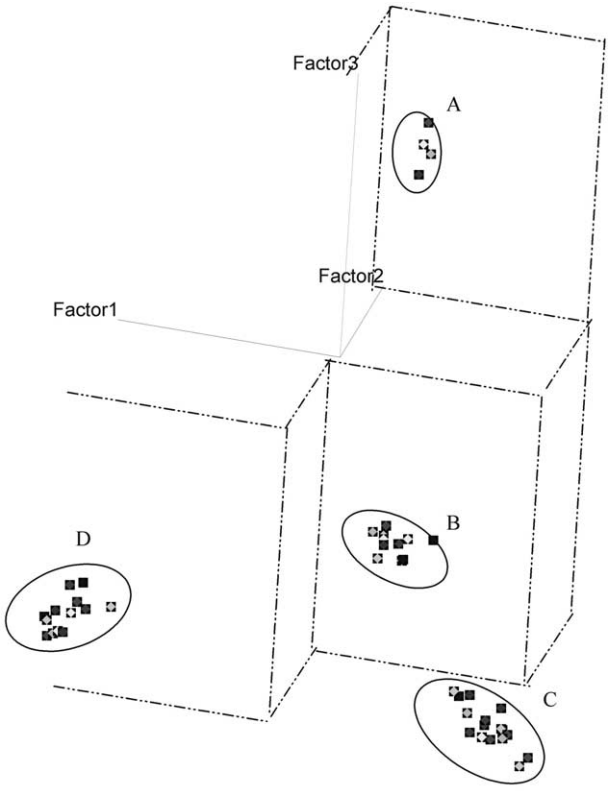
of either COMT or CCoAOMT on phenolic levels in leaves; the profiles of the transgenic lines appeared identical to those of the control line (Fig. 2). To address whether there are statistically significant changes in the profiles that are not immediately apparent to the eye, and to further assess whether OMT down-regulation in stems leads to phenolic profiles that can be discriminated from those corresponding to wild-type plants, we employed statistical data mining tools. As shown below, the entire chromatographic data recorded at different wavelengths were used in Principal Component Analysis (PCA). Although a variable wavelength UV detector has higher sensitivity (Graham, 1991b), a diode array detector was used here to monitor metabolite elution.

Exploratory algorithms such as PCA are designed to reduce the number of dimensions of complex data, by producing a new set of uncorrelated variables (factors) that are linear combinations of the original variables. A plot created with axes in the order of the amount of variance in the data provides a rapid means of visualizing similarities or differences (Fiehn et al., 2000; Sumner et al., 2002). To perform PCA analysis, a data set of peak areas of selected peaks from HPLC profiles can be used. However, for the analysis of complex metabolic profiles, it is very difficult to select an optimized set of integration parameters due to the unavoidable changes of analytical conditions and drift of chromatograms, and manual integration is often time consuming and partly subjective. Nielsen et al. (1998) have introduced an alternative alignment method that uses entire chromatographic data matrices. Instead of a few dozen integrated peaks from one chromatogram, about 16,000 data points from a three wavelengths data matrix were used here for PCA analysis. Fig. 3 shows PCA pattern recognition of 91 chromatograms of soluble phenolic profiles including 46 profiles from leaf extracts and 45 profiles from stem extracts from wild type alfalfa cv Regen SY, COMT and CCoAOMT down-regulated plants in the Regen SY genetic background, and wild-type plants of a different alfalfa cultivar, C349. The latter was included to test the discriminatory power of PCA analysis of phenolic profiles between cultivars in the absence of lignin pathway modification. For each plant line, four to five samples were extracted and duplicate or triplicate injections were made for each extract. In Fig. 3, factor 1 ($>44\%$ variance) indicates that the largest discrimination is between leaves and stems. Since these tissues exhibit unique functional and biosynthetic roles, the separation is expected. While factors 2 and 3 represent much less information in the presence of factor 1, as indicated by their variance of 6.99 and 5.44 respectively, their separation in three-dimensional space indicates their relationship to each



	Variance	Percent	Cumulative
Factor1	289515.19	44.51	44.51
Factor2	45534.57	6.99	51.51
Factor3	38640.25	5.94	57.45

Fig. 3. Principal component analysis of soluble phenolics data using entire HPLC chromatographic data matrices of leaves and stems from four genotypes measured at three wavelengths (254, 280, 310 nm): A, stem (alfalfa C349); B, stem (control); C, stem (COMT down-regulated); D, stem (CCoAOMT down-regulated); E, leaf (control, COMT and CCoAOMT down-regulated); F, leaf (alfalfa C349).



	Variance	Percent	Cumulative
Factor1	34252.121	19.60	19.60
Factor2	20421.429	11.69	31.29
Factor3	18319.406	10.48	41.78

Fig. 4. Principal component analysis of soluble phenolics data using entire HPLC chromatographic data matrices of stems from four genotypes measured at three wavelengths (254, 280, 310 nm): A, alfalfa C349; B, control; C, COMT down-regulated; D, CCoAOMT down-regulated.

other. Factor 2 provides the separation between leaf and stem tissue, while factor 3 separates stem tissues. Factor 3 further indicates that the altered metabolism resulting from COMT down-regulation and CCoAOMT down-regulation of stem tissue is providing separation of these tissues from the control in space. From this plot, CCoAOMT plants exhibit the most dramatic changes. In Fig. 4, stem tissues were re-analyzed using PCA while excluding leaf tissue. Factor 1 (19.60% variance) separates transgenic from control. Once again we see that COMT down-regulation and CCoAOMT down-regulation of stem tissue is providing separation of these tissues from the control in space. From the PCA we are also able to determine that the variance separating the transgenic and control plants (19.60%) is much greater than the separation between these plants and another variety (factor 3, 10.48%). This indicates a significant inter-varietal difference in plant metabolism. PCA analysis could clearly separate the stem phenolic profiles of all lines (Fig. 3), but could not distinguish between plants of the Regen SY cultivar, with or without lignin enzyme modulation, from analysis of leaf profiles. Thus, down-regulation of COMT or CCoAOMT has a readily scoreable effect on stem soluble phenolic profiles, but is phenotypically silent with respect to leaf profiles. However, there are clear differences in leaf profiles between different alfalfa cultivars.

2.3. Wall-bound phenolic profiles of transgenic alfalfa with reduced COMT and CCoAOMT activities

Lignin is a heteropolymer composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) building blocks. The process of lignification varies between different plants and different tissues within a plant. In gymnosperms, the lignin in the tracheary secondary wall mainly consists of guaiacyl units. The secondary walls of fibers and ray cells in angiosperms are guaiacyl–syringyl rich. Grasses contain hydroxyphenyl, guaiacyl, and syringyl units and considerable amounts of covalently linked hydroxycinnamic acids (Anterola and Lewis, 2002). Lignins are heterogeneous polymers with various molecular weights, and can be divided into

"soluble" and "core" lignins. To further characterize lignin modification in the OMT-down-regulated transgenic alfalfa samples used for soluble phenolic profiling, we performed thioacidolysis on both core and soluble lignin fractions from leaf and stem (Table 2). Consistent with our earlier reports (Guo et al., 2000; Marita et al., 2003), it is clear that down-regulation of COMT drastically decreases the S to G ratio in the stem lignin, whereas down-regulation of CCoAOMT slightly increases the S to G ratio. In all the plants, the S/G ratio in the leaf was less than that in the stem. Down-regulation of COMT also causes a dramatic decrease in S/G ratio in lignin from leaves.

The soluble lignins are those molecules of lower molecular weight that can be dissolved in mild alkali solution. Our results show that, compared to the core lignin, there are more syringyl units in the soluble lignin fractions from both stem and leaf (Table 2). The data also show that the effects of COMT or CCoAOMT down-regualtion on S/G ratio are similar in both soluble and core lignin fractions. There are small amounts of H units in the lignin from both leaves (4% of total monomers released by thioacidolysis) and stems (7% of total monomers released). The down-regulation of COMT or CCoAOMT did not affect H lignin levels.

Phenolic compounds play an important role in cross-linking of cell wall components and impact cell wall properties such as enzyme accessibility, extensibility, and digestibility in grasses (Hatfield et al., 1999a; Grabber et al., 2000). These compounds might act to cross-link polysaccharide chains or lignin and polysaccharides. *p*-Coumaric acid has been shown to be mainly esterified to monolignol units as a terminal group of lignin (Ralph et al., 1994; Hatfield et al., 1999a). Ferulic acid is substituted on arabinose or galactose side chains of polysaccharides (Hatfield et al., 1999b). Many of the wall-bound phenolics can be extracted by mild alkali extraction in alfalfa, though it is currently not clear what role they might play in cell wall formation.

Table 3 shows the levels of wall-bound phenolic compounds in COMT and CCoAOMT down-regulated alfalfa plants compared to control plants. For both COMT and CCoAOMT transgenic plants, there were no significant differences in wall-bound phenolic compounds in leaves. In stems, COMT or CCoAOMT down-regulation decreased the amount of wall-bound phenolics but did not change the overall phenolic profiles. When compared to the drastic effect of COMT down-regulation on S lignin levels, it might be concluded that neither COMT nor CCoAOMT is critically involved in the biosynthesis of these wall-bound phenolics, although the down-regulation of the OMTs somehow reduces overall flux into hydroxycinnamic acid metabolism (ie *p*-coumaric acid as well as ferulic acid). A recent study has reported that down-regulation of COMT in maize results in lower *p*-coumaric acid levels but has no effect on ferulic acid (Piquemal et al.,

Table 2
Composition of core and soluble lignin from leaf and stem of control and COMT or CCoAOMT down-regulated alfalfa determined by thioacidolysis. Each measurement represents the mean of two analyses with individual values varying by $<3.3\%$ from mean

			H (μmol/g)	G (μmol/g)	S (μmol/g)	H/G	S/G	H/S
		Control	2.84	61.36	10.58	0.04	0.17	0.26
	Leaf	CCoAOMT	2.27	39.67	7.51	0.05	0.18	0.3
		COMT	2.4	49.04	2.43	0.04	0.04	0.98
Core		Control	21.98	206.22	115.86	0.1	0.56	0.18
	Stem	CCoAOMT	21.95	158.82	108.46	0.13	0.68	0.2
		COMT	18.7	176.06	13.44	0.1	0.07	1.39
		Control	0.37	3.44	1.78	0.1	0.51	0.21
	Leaf	CCoAOMT	3.23	14.39	6.21	0.22	0.43	0.52
		COMT	0.1	6.16	0.16	0.01	0.02	0.65
Soluble		Control	0.36	33.88	27.17	0.09	0.8	0.12
	Stem	CCoAOMT	9.33	38.86	49.76	0.24	1.28	0.18
		COMT	3.42	37.47	5.45	0.09	0.14	0.62

Table 3
Quantification of wall-bound phenolics extracted from cell walls of control and COMT or CCoAOMT down-regulated alfalfa plants by mild alkaline hydrolysis. (expressed as nmol/g dry tissue) ($n=6$)

	Stem					Leaf	
	Vanillic acid	Vanillin	*p*-Hydroxy benzoic acid	*p*-Coumaric acid	Ferulic acid	*p*-Coumaric acid	Ferulic acid
CONTROL	110.37±8.08	168.32±14.65	156.31±10.57	128.83±9.38	164.73±17.45	3501.69±308.29	2730.72±257.99
COMT	89.08±4.87	145.38±6.046	125.61±9.84	105.81±8.22	145.11±15.5	3334.05±103.25	2567.22±86.41
CCoAOMT	80.76±5.53	89.71±8.87	196.85±23.38	123.84±19.43	152.94±17.04	3745.97±142.66	2945.2±114.16

2002). It has recently been suggested that ferulic acid originates from the hydrolysis of feruloyl CoA via a putative feruloyl esterase (Sancho et al., 1999; Piquemal et al., 2002), placing CCoAOMT as a critical enzyme in ferulic acid biosynthesis. However, in our CCoAOMT down-regulated alfalfa, there was no dramatic decrease in ferulic acid content. The biosynthetic origin of the methylated hydroxycinnamic acids may be less certain than previously thought. Alternatively, these compounds may be produced in different cells from those targeted for COMT or CCoAOMT down-regulation by the *PAL* promoter, and the small amount of residual enzyme activity may be localized to the sites of ferulic acid synthesis.

2.4. Conclusions

In summary, the analytical procedures described here allow for the identification and quantification of soluble phenolics, wall-bound phenolics and lignins from the same samples. HPLC profiling of methanolic extractions from alfalfa stems coupled with PCA analysis provides fingerprints for discriminating genetically modified plants from wild-type plants, and can also distinguish between different cultivars (which exhibit greater differences in their leaf phenolic profiles than do the COMT or CCoAOMT down-regulated transgenic plants compared to their parent non-transformed line). The procedure is rigorous and reproducible and can be automated. Within the detection limits of the method, we could not show the accumulation of any proposed intermediate in monolignol biosynthesis in the soluble fraction from transgenic alfalfa stems down-regulated in COMT or CCoAOMT. Our results indicate that, although COMT down-regulation leads to a drastic decrease in S lignin biosynthesis, and CCoAOMT is believed to be essential for introduction of the 3-methoxyl group into monolignols, down-regulation of neither OMT had a strong effect on levels of wall-bound ferulic acid.

Metabolic profiling is a powerful tool for detailed phenotyping of transgenic plants, and is likely to become a requirement for determination of ''substantial equivalence'' (to wild-type) of transgenic cultivars developed for commercialization such as COMT and CCoAOMT down-regulated alfalfa plants which have improved forage digestibility.

3. Experimental

3.1. Materials

Chemicals and solvents were obtained from Sigma (St Louis, MO, USA). Analytical grade chemicals were used and all solvents used in sample preparation and HPLC were of HPLC grade. Water was generated from a Milli-Q deionization system (Millipore, MA, USA).

3.2. Plant material, growth conditions and sample collection

Alfalfa (*Medicago sativa* cv Regen SY and C349) plants were grown in the greenhouse under standard conditions with a 16-h day from 6:00h to 22:00h facilitated by supplementary lighting. The average temperature was 21 °C, and humidity ranged from 58 to 87% RH.

All genetic transformations were performed with clonally propagated material of one selected highly regenerable alfalfa line of cv Regen SY. The generation of transgenic alfalfa lines, determination of transgene insertion patterns, characterization of lignin composition, and assay of enzyme activity have been reported previously (Guo et al., 2000). Vegetatively propagated plants were cut back in April 2002. The plants were collected when stems had produced eight internodes. The leaves and stems from internodes three to seven were separated and frozen in liquid nitrogen. The samples were ground into a powder with a mortar and pestle under liquid nitrogen and freeze dried.

3.3. Extraction of soluble phenolic compounds

Extractions of freeze-dried material of alfalfa stem or leaf samples (50 mg) were carried out in screw-capped Pyrex test tubes (4 ml). Methanol (0.8 ml) and hexane (0.8 ml) were added to the tubes and samples extracted by sonication for 30 min in a Branson 2510 ultrasonic cleaner (Branson Ultrasonic Corporation, Banbury, CT USA). Then 0.3 ml water was added and the samples sonicated for a further 30 min. After brief centrifugation at 3000 *g* at room temp, the hexane and aqueous phases were separated with a microsyringe. The aqueous fractions were used directly for soluble phenolic profiling.

3.4. Extraction of wall-bound phenolic compounds

The residue after methanol:hexane extraction was washed sequentially with 70 and 100% aqueous methanol (3 ml). After removing the methanol, the samples were dried at 37 °C and weighed. Aqueous 1 N NaOH (1.6 ml) was added to the tubes and the samples extracted overnight at room temp in the dark. The solvents were separated by centrifugation and the samples extracted again with 1 N NaOH (1.6 ml) for 6 h and then washed with water (1.6 ml). The combined aqueous extracts were acidified to pH 1∼2 with 6 N HCl and extracted three times with an equal volume of ethyl acetate. The combined organic phases were dried under a stream of nitrogen and re-suspended in 70% methanol for HPLC analysis. The identification of wall-bound

phenolics was made by comparing the UV spectra and retention times with those of the authentic compounds. Quantification of wall-bound phenolics was made by external standard method. The precipitated residue in the acidified aqueous layer was washed with a small amount of water, dried at 37 °C and used for determination of the composition of soluble lignin.

3.5. Lignin analysis

After extraction of wall-bound phenolic compounds, the tissue samples were washed with water and methanol twice separately, and then dried at 37 °C. The samples were weighed and used for lignin analysis. Thioacidolysis was used to determine the composition of cell wall and soluble lignins as described by Lapierre et al. (1986). Lignin monomer derivatives were analyzed by GC/MS.

3.6. HPLC and GC

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 XTerra 5 μ reversed phase column (5 μm particle, 250 × 4.6 mm) was used, with the mobile phase consisting of 10 mM ammonium formate in water (pH 3.7) (A), and 10 mM ammonium formate in acetonitrile/water (80:20) (B). The gradient used was 90% A, 10% B for 2 min, a linear gradient to 65% A, 35% B in 50 min, then a linear gradient to 100% B in 10 min. A flow rate of 1 ml min^{-1} was used in all experiments. LC/MS was used to identify the metabolites of interest and all mass spectra were acquired using a Bruker Esquire ion-trap mass spectrometer equipped with an electrospray ionization source (column: JT Baker octadecyl, 5 μm particle, 250 × 4.6 mm. Solvent: A 0.1% acetic acid in water, B acetonitrile. Gradient: 8% B–30% B in 50 min). GC/MS was performed on a Hewlett Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60 m × 0.25 mm × 0.25 mm film thickness) using electron ionization (70 eV).

3.7. Statistical analysis

Statistical analysis was performed using SAS programs (SAS Institute, Cary, NC). Chromatogram alignment for HPLC profiles was performed using Align program (Duran et al., 2003) and CowTool program (Nielsen et al., 1998). PCA was performed using Pirrouette software (Informetrix, Woodinville, WA). The data sets collected at three wavelengths (254, 280, and 310 nm) were used as input data for PCA.

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