Reduced Lignin Content and Altered Lignin Composition in Transgenic Tobacco Down-Regulated in Expression of \( \text{l-Phenylalanine Ammonia-Lyase or Cinnamate 4-Hydroxylase} \)


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We analyzed lignin content and composition in transgenic tobacco (\textit{Nicotiana tabacum}) lines altered in the expression of the early phenylpropanoid biosynthetic enzymes \textit{L-phenylalanine ammonia-lyase} and cinnamate 4-hydroxylase ( \( \text{C4H} \)). The reduction of \( \text{C4H} \) activity by antisense expression or sense suppression resulted in reduced levels of Klason lignin, accompanied by a decreased syringyl/guaiacyl monomer ratio as determined by pyrolysis gas chromatography/mass spectrometry. Similar reduction of lignin levels by down-regulation of \textit{l-phenylalanine ammonia-lyase}, the enzyme preceding \( \text{C4H} \) in the central phenylpropanoid pathway, did not result in a decreased syringyl/guaiacyl ratio. Rather, analysis of lignin methoxyl content and pyrolysis suggested an increased syringyl/guaiacyl ratio. One possible explanation of these results is that monolignol biosynthesis from \textit{l-phenylalanine} might occur by more than one route, even at the early stages of the core phenylpropanoid pathway, prior to the formation of specific monolignol precursors.

There is currently intense interest in modifying the content and/or composition of the cell wall structural polymer lignin as a means of improving the efficiency of the paper pulping process for forest trees or of increasing digestibility of forages for ruminant animals (Whetten and Sederoff, 1991; Boudet and Grima-Pettenati, 1996; Campbell and Sederoff, 1996).

Recent studies have concentrated on attempts to down-regulate the levels of enzymes involved in the reactions specific for lignin monomer synthesis by expression of homologous or heterologous antisense genes in transgenic plants (Dwivedi et al., 1994; Halpin et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Van Doorsselaere et al., 1995; Sewalt et al., 1997). Although the biosynthetic pathway to lignin monomers is relatively well understood, involving consecutive hydroxylation and O-methylation reactions leading from \textit{p-coumaric acid} via ferulic acid (the monomethoxylated precursor of the \( \text{G} \) residues of lignin) to syringic acid (the dimethoxylated precursor of the \( \text{S} \) residues of lignin), it has recently been suggested that parallel pathways of monomer hydroxylation and methylation could occur at the level of the CoA thioesters (Ye et al., 1994) or even at the level of the aldehydes formed after the first reduction of the CoA thioesters (Matsui et al., 1994; Fig. 1).

The existence of a metabolic grid for the O-methylation of monolignols would complicate the interpretation of experiments in which a single enzyme of the pathway was down-regulated. Indeed, several reports of the effects of antisense inhibition of enzymes involved in the late reactions of monolignol biosynthesis have presented unpredictable and sometimes contradictory results. Ni et al. (1994) reported that modest down-regulation of COMT activity in transgenic tobacco (\textit{Nicotiana tabacum}) leads to a small reduction in lignin content with no significant change in lignin composition. However, other groups have shown that strong down-regulation of COMT in tobacco or poplar (\textit{Populus tremula} \( \times \) \textit{Populus alba}) leads to a drastic reduction in \( \text{S} \) units, with corresponding incorporation of 5-hydroxy \( \text{G} \) units into lignin, the overall level of which is not reduced (Atanassova et al., 1995; Van Doorsselaere et al., 1995). The latter phenotype is similar to that reported for certain brown-midrib mutants with increased forage digestibility, and the \( \text{Bm3} \) mutation in maize (\textit{Zea mays}) and sorghum

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Abbreviations: CAD, cinnamyl alcohol dehydrogenase; \( \text{C4H} \), cinnamate 4-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; \( \text{G} \), guaiacyl; NDF, neutral detergent fiber; PAL, \textit{l-Phenylalanine ammonia-lyase}; \( \text{S} \), syringyl.
(Sorghum bicolor) has recently been shown to be in the \textit{COMT} structural gene (Vignols et al., 1995).

To engineer plants with agronomically useful lignin-related traits, it will be necessary to devise strategies that can flexibly and predictably yield reductions in lignin content and/or changes in lignin monomer composition. Because most reports suggest that reduced expression of the late enzymes of lignin monomer synthesis, \textit{COMT} and \textit{CAD}, affects lignin composition without affecting content, it may be necessary to reduce the flux into the lignin pathway at an earlier stage to reduce lignin content. It has recently been demonstrated that decreases in Phe pool size (Jones et al., 1995; Yao et al., 1995) or reduced activity of \textit{PAL}, the entry enzyme into the phenylpropanoid pathway (Elkind et al., 1990; Bate et al., 1994), leads to decreased lignin content in transgenic plants. However, there are no reports to date of the effects of such manipulations on lignin composition.

We describe the composition of lignin from transgenic tobacco plants with severely reduced lignin levels due to down-regulation of \textit{PAL} or \textit{C4H} activities. A reduction in \textit{PAL} levels leads to an increase in the S/G ratio, whereas reduced \textit{C4H} activity leads to a decrease in the S/G ratio. These observations support the existence of some sort of metabolic channeling between the enzymes of the central phenylpropanoid pathway and those of monolignol biosynthesis and provide a basis for the development of new strategies for lignin modification to improve digestibility of forage crops.

MATERIALS AND METHODS

Transgenic tobacco (\textit{Nicotiana tabacum} L. cv Xanthi) plants originated from two independent sets of internally controlled experiments. For both sets of transgenic plants (\textit{PAL}, \textit{C4H}, and their respective controls), all plants were grown together under exactly the same environmental conditions and were harvested at the same time and physiological stage. \textit{PAL}-modified and control transgenic plants were grown from seed and harvested after 5 weeks. \textit{C4H} transgenic plants and corresponding controls were primary transformants cut back simultaneously and harvested after 4 weeks of regrowth.

\textit{PAL} lines evaluated were severely \textit{PAL} sense-suppressed (160P3, second-generation progeny carrying a bean [\textit{Phaseolus vulgaris}] \textit{PAL} transgene in the sense orientation), \textit{PAL}-suppressed but recovering (274-T5 fifth-generation selfed progeny), \textit{PAL}-overexpressing (YEIO-6T1, first-generation selfed progeny), or operationally wild type (C17, first-generation progeny line that had lost the bean \textit{PAL} transgene through segregation and therefore displayed a wild-type \textit{PAL} phenotype), as described by Bate et al. (1994) and Howles et al. (1996). \textit{C4H} lines were primary transformants carrying either an empty vector or an alfalfa (\textit{Medicago sativa}) \textit{C4H} transgene (Fahrendorf and Dixon, 1993) in the sense or antisense orientation, resulting in independent transformants with either normal, suppressed, or increased \textit{C4H} activity. Designation of sense suppression was based on the presence of alfalfa \textit{C4H} transcripts but reduced overall \textit{C4H} enzymatic activity compared with the average and SD of values from a population of 15 control plants (Fig. 2).

Enzyme Extraction and Assays

Midstem sections (internodes 10 and 11 for \textit{PAL} plants, internodes 8–11 for \textit{C4H} plants, counting from the first fully opened leaf at the top) were collected and ground under liquid N$_2$. Powdered tissue was divided into two tubes, one for assay of enzyme activities and the other for lignin analysis, and stored at $-70^\circ$C.

\textit{PAL} (cytosolic) and \textit{C4H} (microsomal) activities were assayed by the methods described by Edwards and Kessmann (1992).
Histochemical Analysis

The 10th internode from tobacco plants with selected PAL or C4H phenotypes was collected in a second sampling from regrown plants. Sections obtained by freehand sectioning were stained for lignin using phloroglucinol-HCl or the Mäule color reaction according to the method of Nakano and Meshitsuka (1992). Phloroglucinol-stained sections were photographed within 30 min. In addition, sections were stained according to the method of Srebotnik and Messner (1994) with 0.1% aqueous safranin-O (color index no. 50240; Sigma) and then with 1% aqueous astrablue (Sigma) for 3 min each.

Statistical Analysis

Differences in enzymatic activity and lignin characteristics between groups of control and genetically modified plants were examined in internally controlled experiments by one-way analysis of variance (Snedecor and Cochran, 1989) using the data analysis tools in the program Excel (Microsoft, Redmond, WA). In the PAL experiment seedlings of independent transformants and control lines were used as replicates (Table III). In the C4H experiment independent primary transformants were grouped into low (antisense/sense-suppressed) and high (control/overexpressor) C4H activity classes.

RESULTS

Transgenic Tobacco Plants with Altered Expression of Core Phenylpropanoid Pathway Enzymes

The generation of transgenic tobacco plants with altered levels of C4H expression, using binary vector constructs containing the complete alfalfa C4H cDNA sequence (Fahrendorf and Dixon, 1993) in both sense and antisense orientations, will be described in more detail elsewhere (J.W. Blount, S.A. Masoud, K.L. Korth, V.J.H. Sewalt, T. Fahrendorf, and R.A. Dixon, unpublished data). Plants transformed with the antisense construct expressed low levels of C4H transcripts and had significantly reduced C4H activity as initially determined in leaf tissue (approximately 35% of wild type on average). Plants transformed with the sense construct fell into two classes: overexpressors (470% of wild-type activity on average) and plants exhibiting reduced (45% of wild type on average) levels of C4H activity. The latter are designated as operationally sense-suppressed plants, although it should be cautioned that the exact mechanism for the reduced C4H activities has not been determined. The molecular and biochemical phenotypes of a subset of the C4H transformants that were selected for analysis of lignin content and composition are summarized in Table I. Further details of the phenotypes of the C4H transgenic plants will be described elsewhere (J.W. Blount, S.A. Masoud, K.L. Korth, V.J.H. Sewalt, T. Fahrendorf, and R.A. Dixon, unpublished data).

We previously generated transgenic tobacco plants in which the level of PAL activity was severely reduced as a result of sense suppression arising from the genomic inte-
first analyzed a population of 15 independent control near-isogenic tobacco lines have been generated with a extractable lignin (Bate et al., 1994). Another sense- selfed generations (Bate et al., 1994). Plants with very low wide range of PAL activities, and representative indepen- dent plants from these various classes were chosen for suppression phenotype was gradually lost in successive plants for C4H activity, PAL activity, NDF (pectin-free cell wall material), and Klason lignin levels in stem tissue. The results shown in Figure 2 demonstrate that the natural variation in C4H and PAL activity in stems was characterized by sds of ±22 and ±26% of the mean, respectively. Means for NDF and Klason lignin had an sd of ±6% each. 

**Table I. Characteristics of plants used for analysis of effects of modified C4H expression on lignin content and composition**

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>Construct</th>
<th>Transgene Copy No.</th>
<th>C4H Transcript Level</th>
<th>C4H Activity in Stems</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>11A</td>
<td>Untransformed</td>
<td>-c</td>
<td>ND</td>
<td>117</td>
<td>Control</td>
</tr>
<tr>
<td>8A</td>
<td>Untransformed</td>
<td>-</td>
<td>ND</td>
<td>83</td>
<td>Control</td>
</tr>
<tr>
<td>2C</td>
<td>Sense</td>
<td>1</td>
<td>++</td>
<td>66</td>
<td>Sense-suppressed</td>
</tr>
<tr>
<td>32C</td>
<td>Sense</td>
<td>ND</td>
<td>+ +</td>
<td>60</td>
<td>Sense-suppressed</td>
</tr>
<tr>
<td>25C</td>
<td>Sense</td>
<td>+</td>
<td>+ + +</td>
<td>39</td>
<td>Sense-suppressed</td>
</tr>
<tr>
<td>72B</td>
<td>Antisense</td>
<td>+ 1</td>
<td>+</td>
<td>42</td>
<td>Antisense</td>
</tr>
<tr>
<td>13B</td>
<td>Antisense</td>
<td>+ 1</td>
<td>+</td>
<td>24</td>
<td>Antisense</td>
</tr>
<tr>
<td>201C</td>
<td>Sense</td>
<td>+ 1</td>
<td>+ + + +</td>
<td>214</td>
<td>Overexpressor</td>
</tr>
</tbody>
</table>

* Transgene copy number was determined by Southern analysis using the alfalfa C4H-coding sequence as a probe and segregation of kanamycin resistance in the T₁ generation. The C4H transcript level was determined by northern analysis using the alfalfa C4H-coding sequence as a probe. c, No transgene. d ND, Not detected. e, Barely detectable. f, Intermediate level. g, +++, Strong expression. h, ++++, Very strong expression.

**Lignin Content and Composition in Transgenic Tobacco with Reduced or Increased Expression of C4H**

The effects of alterations in C4H activity caused by expression of the alfalfa C4H transgene in the sense or antisense orientations on NDF, Klason lignin, and lignin methoxyl content are shown in Table II. In the set of eight independent plants analyzed, overexpression resulted in approximately twice the wild-type C4H activity in stem tissues (similar to the increase in PAL in PAL-overexpressing lines [Howles et al., 1996]; see below), whereas the strongest antisense effect reduced C4H activity to approximately 20% of wild-type levels. Overexpression of C4H had no effect on NDF, Klason lignin, or lignin methoxyl content measured by wet chemistry, whereas down-regulation to less than 50% of wild-type levels significantly reduced NDF and lignin levels in most lines (Table II), the latter to as much as below 20% of wild type (expressed as a percentage of dry matter) in the most severely down-regulated line (Fig. 3). The only anomalous result from the plants analyzed in Table II was from line

**Table II. Relationship among C4H activity, NDF, Klason lignin, and lignin methoxyl group content in transgenic tobacco with modified C4H expression**

<table>
<thead>
<tr>
<th>Plant No.</th>
<th>Status*</th>
<th>C4H nkat/g</th>
<th>C4H Mean ± sd for set</th>
<th>NDF % Dry matter</th>
<th>NDF Mean ± sd for set</th>
<th>Klason Lignin % NDF</th>
<th>Klason Lignin Mean ± sd for set</th>
<th>Lignin Methoxyl Group % Klason lignin</th>
<th>Lignin Methoxyl Group Mean ± sd for set</th>
</tr>
</thead>
<tbody>
<tr>
<td>201C</td>
<td>OE</td>
<td>8.93</td>
<td>27.22</td>
<td>9.34</td>
<td>15.36</td>
<td>8.60</td>
<td>14.90</td>
<td>15.74 ± 1.08</td>
<td></td>
</tr>
<tr>
<td>11A</td>
<td>C</td>
<td>4.86</td>
<td>30.97</td>
<td>9.34</td>
<td>16.95</td>
<td>14.90</td>
<td>14.42</td>
<td>15.74 ± 1.08</td>
<td></td>
</tr>
<tr>
<td>8A</td>
<td>C</td>
<td>3.48</td>
<td>28.26</td>
<td>9.20 ± 0.54</td>
<td>14.22</td>
<td>14.90</td>
<td>13.30 ± 1.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C</td>
<td>SS</td>
<td>2.76</td>
<td>31.38</td>
<td>11.08</td>
<td>14.42</td>
<td>14.90</td>
<td>13.30 ± 1.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32C</td>
<td>SS</td>
<td>2.49</td>
<td>21.76</td>
<td>7.95</td>
<td>12.04</td>
<td>14.90</td>
<td>13.30 ± 1.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72B</td>
<td>AS</td>
<td>1.73</td>
<td>20.32</td>
<td>6.49</td>
<td>15.34</td>
<td>14.90</td>
<td>13.30 ± 1.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25C</td>
<td>AS</td>
<td>1.64</td>
<td>20.09</td>
<td>7.62</td>
<td>10.51</td>
<td>14.90</td>
<td>13.30 ± 1.97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* OE, Overexpressor; C, empty vector control; SS, sense-suppressed; AS, antisense.
Lignin Alterations in Transgenic Plants

2C, in which slightly reduced C4H activity was accompanied by an increase in NDF and Klason lignin levels.

Increasing C4H activity above wild-type levels had no effect on the S/G ratio as determined by pyrolysis GC-MS analysis, whereas, surprisingly, reduction to approximately 40% of wild-type levels caused a large reduction to approximately 0.05 in antisense line 72B (Figs. 4 and 5). This resulted from a drastic decrease in S residues accompanied in the samples with the lowest lignin levels by a smaller decrease in G residues. In contrast, methoxyl analysis by wet chemistry suggested more modest reductions in methoxyl content as a percentage of Klason lignin level in the down-regulated lines. Because of low levels of free iodine in the hydriodic acid used in the iodometric determination of methoxyl content, measurement by this method becomes less reliable at very low lignin levels.

C4H-down-regulated plants displayed reduced phloroglucinol staining compared with the respective controls (Fig. 6, a and b), consistent with the reduction in Klason lignin. Mäule staining of C4H-reduced plants showed a change in color from wine-red to dark-brown (Fig. 6, e and f), which is indicative of a reduction in S content. Safranin-O, a basic dye, stains lignin red. Astra-blue, a phthalocyanin dye, is incorporated into cellulose fibers,
Lignin Content and Composition in Transgenic Tobacco with Reduced or Increased Expression of PAL

In Table III the PAL activities, NDF, Klason lignin, and lignin methoxyl content of stem tissue from 11 independent transformants representative of four classes of PAL expression are shown: wild type (C17, a line that lost the bean PAL transgene, and therefore the sense suppression phenotype, through segregation), PAL-suppressed (160P3, T2 generation), PAL-suppressed but recovering (274-T5, fifth-generation selfed progeny of a strongly sense-suppressed primary transformant), and overexpressors (first-generation selfed progeny of line YE10-6). Although there was significant variation in extractable PAL activity within each class, the ranges are distinct and represent clearly defined PAL phenotypes. Reduction in PAL activity in the most severely sense-suppressed lines caused a large decrease in Klason lignin content and a corresponding effect on NDF value in two of the three plants analyzed. At the same time, however, there was a significant increase in methoxyl group content in the two most severely PAL-suppressed plants. The increased methoxyl content relative to total lignin amount in the plants with reduced lignin levels suggests that inhibition of the flux into the phenylpropanoid pathway has qualitative as well as quantitative effects on lignin synthesis.

The effect of altered PAL activity on lignin composition was confirmed by pyrolysis GC-MS (Fig. 7). In the control C17 line the S/G ratio was 1.102, which is comparable to that in untransformed control lines (data not shown). In the strongly PAL-suppressed line, pyrolysis GC-MS showed corresponding to G units decreased, whereas the level of S units remained relatively unaffected, resulting in an increase in the S/G ratio to approximately 1.9. In contrast, overexpression of PAL had little effect on the S/G ratio, consistent with the lack of any significant change in lignin levels. Analysis of additional PAL-suppressed lines by pyrolysis GC-MS confirmed the above findings, with S/G staining them blue only in the absence of lignin. Dual staining with safranin-O and astra-blue confirmed the above results; C4H-suppressed plants showed distinct rows of blue cell walls protruding from the pith into the vascular region (ray cells) or patches of blue within the vascular region, which may be an indication that the antisense or sense-suppression effects are not evenly distributed over different cell types (Fig. 6, g and h).

Figure 5. Relation between C4H activity and S/G ratio of Klason lignin from stems of a range of transgenic tobacco plants harboring alfalfa C4H constructs or untransformed control plants of the same physiological stage. •, Control plants; ■, antisense and sense-suppressed plants; and ○, overexpressor plants.

Figure 6. Histochemical analysis of lignin in transgenic tobacco lines. Cross-sections of stems of transgenic tobacco lines (10th internode from top) stained with phloroglucinol-HCl (A–D), Mäule reagent (E and F), and safranin-O and astra-blue (G–J). A, Untransformed control for C4H transgenics (11A); B, C4H sense-suppressed transformant (32C); C, PAL overexpressor (10-6); D, PAL sense-suppressed (160-P3); E, untransformed control for C4H transgenics (11A); F, C4H sense-suppressed (32C); G, untransformed control for C4H transgenics (11A); H, C4H sense-suppressed (32C); I, PAL control line (105C); J, PAL sense-suppressed (160-P3). Magnification, X25 (G–J) and X50 (A–F).
The very poor staining of PAL-suppressed tobacco stem sections with phloroglucinol confirmed the reduction in lignin content (Fig. 6d), whereas PAL-overexpressing plants stained strongly (Fig. 6c). PAL suppression resulted in less intense Mäule staining than in plants with wild-type PAL levels and a slight color shift from plain brown (indicative of predominance of G lignin in the vascular tissue of wild-type tobacco) to a patterned wine-red/brown staining (indicative of a shift to S lignin in xylem ray cells and/or sclerenchyma fibers in PAL-suppressed plants; data not shown). Double staining with safranin-O and astrablue confirmed the results obtained with phloroglucinol; shifts from red to purple or blue in the vascular ring in plants with reduced PAL activity were indicative of reduced lignin level and increased accessibility of cellulose to the stain (Fig. 6, i and j).

**DISCUSSION**

**Genetic Manipulation of Lignin Content in Transgenic Tobacco**

We are attempting to develop genetic engineering strategies for reducing lignin content to improve digestibility of forage species. However, several reports of targeted down-regulation of late enzymes of lignin biosynthesis have failed to demonstrate reductions in lignin content (Dwivedi et al., 1994; Halpin et al., 1994; Atanassova et al., 1995; Van Doorsselaere et al., 1995), highlighting our lack of understanding of the regulatory control points for monolignol biosynthesis (Lewis and Yamamoto, 1990).

In the case of down-regulation of CAD, the last enzyme in the classical monolignol pathway, reducing the formation of hydroxycinnamyl alcohols results in the incorporation of the corresponding aldehydes into lignin, leading to a wine-red lignin with increased extractability (Halpin et al., 1994; Higuchi et al., 1994; Hibino et al., 1995; Bernard-Vailhé et al., 1996). Reduction in the activity of the bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase in transgenic plants to less than 10% of wild-type levels resulted in qualitative changes in lignin composition (reduced S/G ratio), with no apparent effect on overall lignin content (Atanassova et al., 1995; Van Doorsselaere et al., 1995).

The potential complications of a metabolic grid associated with the ring substitution reactions in the later stages of monolignol synthesis would at first sight make the approach of reducing lignin by limiting flux at an earlier stage in the pathway more attractive, in spite of the ultimate need to engineer such modifications under tight temporal and spatial control to avoid pleiotropic effects such as the increased disease susceptibility observed in plants with reduced PAL activity (Maher et al., 1994; Pallas et al., 1996). Our preliminary results with PAL-suppressed plants indicated a reduction in lignin based on toluidine blue staining (Elkind et al., 1990), and this was later confirmed by analysis of thioglycolic acid lignin levels in a series of progeny lines representative of the wide variation in PAL activity levels (Bate et al., 1994).

We have now shown that reduction of PAL activity to approximately 15% of wild-type levels gives an approximately 2-fold decrease in Klasson lignin as a percentage of...
Reducing the level of C4H activity in tobacco stems results in a corresponding decrease in PAL activity (J.W. Blount, S. Masoud, and R.A. Dixon, unpublished results). However, the reduced lignin levels in C4H transgenic plants are unlikely to be simply the result of the reduced PAL activity, because of the difference in effect on lignin composition. Furthermore, the PAL activity (measured in the supernatant of the same extract used for determination of C4H activity) in stem tissues of various C4H-suppressed lines (e.g. 43.4 nkat/g protein for line 13B) combined with wild-type C4H activity would not, on the basis of the data shown in Table III, be predicted to give a lignin reduction of the magnitude observed.

Genetic Manipulation of Lignin Composition in Transgenic Tobacco

Severely PAL-suppressed plants produced low levels of lignin with increased methoxyl content and S/G ratio indicative of a reduction in G lignin. Contrary to the situation with severe PAL suppression, the S/G ratio was reduced by C4H suppression, indicating that the altered S/G ratio in C4H-down-regulated plants was the result of the change in C4H activity rather than of the associated decrease in PAL activity observed in these plants (J.W. Blount, S. Masoud, and R.A. Dixon, unpublished results). Overexpression of either enzyme resulted in no change in lignin composition, which is indicative of downstream control points in the lignin biosynthetic pathway.

Critical to the interpretation of the data concerning lignin composition is the nature of the method used to determine the S/G ratio. Because of the complexity and heterogeneity of the lignin polymer, most methods for the determination of lignin composition have some limitations. Pyrolysis MS and pyrolysis GC-MS combine direct depolymerization of organic material by rapid heating in vacuo and visualization of dissociation products (Ralph and Hatfield, 1991). In the case of lignin, dissociation products include monomeric, dimeric, and trimeric structural elements.

Recently, the use of pyrolysis GC-MS has become routine in agricultural chemistry and plant biology research (Mulder and Emons, 1993; Niemann et al., 1993; Boudet et al., 1995) to elucidate differences between lignin assays (Reeves and Galletti, 1993; Hatfield et al., 1994) and for characterization of tobacco lignin in wild-type (Faix et al., 1992) and transgenic plants (Halpin et al., 1994; Sewalt et al., 1997). Pyrolysis is a unique and rapid lignin-fingerprinting tool capable of determining lignin monomer composition, but it lacks the capability of thioacidolysis, a commonly used method of lignin analysis, to provide detailed information about lignin-bonding patterns and functionality (Boudet et al., 1995). However, thioacidolysis specifically targets β-O-4 linkages in uncondensed lignin moieties (Lapierre et al., 1985). A direct comparison of the two methods can be drawn from the results of Halpin et al. (1994), who reported a sharp decrease in the S/G ratio in CAD-antisense tobacco from 0.83 (control) to 0.46 as determined by thioacidolysis and a similar decrease in S/G as determined by pyrolysis MS.

We tested the reproducibility of the pyrolysis GC-MS method for tobacco lignin by determining the levels of the G- and S-derived residues in five independent control lines (of the cv Xanthi type used in the PAL-suppression experiments). The values (in arbitrary units relative to the internal standard) were 2.574 ± 0.165 for G units and 2.900 ± 0.320 for S units, giving an S/G ratio for the group of
The procedure is a sensitive and reproducible method for determining the S/G ratio in control and transgenic tobacco lines. Although pyrolysis is a relatively efficient depolymerization method that targets more than just the uncondensed lignin moieties that are not depolymerized by pyrolysis but that are quantified by the Klason lignin method and are also represented in the quantification of methoxyl groups. Such unavoidable difficulties in lignin analysis may explain why the highly down-regulated C4H line 13B shows virtually no G or S residues in the pyrogram (Fig. 4D) but nevertheless still contains approximately 30% of the Klason lignin of wild-type plants. The methoxyl determination, which involves exhaustive demethylation with hydriodic acid (TAPPI, 1972; Zakis, 1994), is most inclusive with regard to different lignin portions. Therefore, the methoxyl value includes the entire residual lignin, which, in the case of C4H suppression, seems to be of a more condensed nature (as judged by the less efficient pyrolysis and only slightly reduced total methoxyl content). PAL suppression results in reduced lignin content, with the remaining lignin being relatively uncondensed (as judged by efficient pyrolysis and increased S/G ratio and methoxyl content).

Our data provide new information about the effects of reduction of flux into phenylpropanoid synthesis on lignin composition. The observation that the reduction of PAL or C4H activities leads to altered lignin composition in addition to reduced lignin levels contrasts with previous observations of altered composition but no change in lignin level following down-regulation of later enzymes in the monolignol pathway (Atanassova et al., 1995; Van Doorsselaere et al., 1995). Furthermore, the apparently opposite effects of PAL and C4H down-regulation on the S/G ratio were totally unexpected. This finding suggests two possibilities: (a) down-regulation of PAL or C4H could lead to differential feed-forward effects on later downstream enzymes (this is not the case for COMT, which is not reduced in C4H-down-regulated plants [V.J.H. Sewalt and R.A. Dixon, unpublished results]) and (b) the route for monolignol formation at the level of the early, “core” reactions of the phenylpropanoid pathway may, like the later reactions of O-methylation and hydroxylation, not be a single linear pathway, although it is unlikely that parallel pathways utilizing enzymes in addition to PAL and C4H exist.

One possible explanation for the different effects of PAL and C4H down-regulation could be that these enzymes are organized into more than one complex or metabolic channel, perhaps also involving later enzymes such as the CoA ligase or O-methyltransferases. Such putative channel complexes might be associated with specific isoforms of PAL, which is encoded by a multigene family in tobacco (Fukasawa-Akada et al., 1996) and most other species studied. Such complexes might direct Phe or cinnamate specifically into the production of G or S units, and partitioning of flux into these complexes might not be equally affected by down-regulation of PAL compared with C4H, particularly if different tobacco PAL forms are differentially sensitive to co-suppression by the bean PAL transgene.

Such hypotheses remain to be tested. However, channeling of Phe through the PAL and C4H reactions has been previously demonstrated by metabolic labeling experiments in microsomes from potato tubers and cucumber and buckwheat hypocotyls (Czichi and Kindl, 1975, 1977; Hrazdina and Wagner, 1985), and we have recently confirmed this in tobacco (S. Rasmussen and R.A. Dixon, unpublished results). It will be interesting to study the effects of PAL down-regulation on lignin composition in monocots, in which the PAL enzyme also has Tyr-ammonia-lyase activity (Rösler et al., 1997) and can therefore bypass the C4H reaction in the synthesis of 4-coumarate.

In conclusion, the present results indicate the feasibility of reducing lignin content for forage improvement, with targeted changes in lignin monomer composition, by transgenic strategies. At the same time, they reveal the possibility of a hitherto unexpected complexity in the functioning of the early stages of the core phenylpropanoid pathway.

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