

Lignification in transgenics deficient in 4-coumarate 3-hydroxylase (C3H) or the associated hydroxycinnamoyl transferase (HCT)

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Abstract

Down-regulation of the gene encoding 4-coumarate 3-hydroxylase (C3H) in angiosperms massively but predictably increased the proportion of *p*-hydroxyphenyl (P) units relative to the normally dominant syringyl (S) and guaiacyl (G) units. Alfalfa stem levels of up to ~65% P (from wild-type (WT) levels of ~1%) resulting from downregulation of C3H were measured by traditional degradative analyses as well as 2D ¹³C–¹H correlative NMR methods. Such levels put these transgenics well beyond the P:G:S compositional bounds of normal plants. NMR also revealed structural differences in the interunit linkage distribution that characterizes a lignin polymer. Less severely elevated P-levels were also detected in C3H-downregulated poplar and in HCT-downregulated *P. radiata* tracheid cultures, but with different structural effects. The compositional and structural changes remain consistent with the existing theory of lignification based on combinatorial radical coupling reactions under simple chemical control. Such structural differences form a basis for explaining differences in digestibility and pulping performance of C3H/HCT-deficient plants.

Introduction

The effects on lignification of downregulating most of the genes for enzymes on the monolignol biosynthetic pathway have been reasonably well studied. The exception is the crucial hydroxylase C3H, and its associated HCT, taking *p*-coumarate to caffeate — the gateway to the major monolignols, coniferyl and sinapyl alcohols.

Arabidopsis Mutant

A C3H-deficient *Arabidopsis ref8* mutant produced no detectable guaiacyl (G) nor syringyl (S) lignin; only *p*-hydroxyphenyl (P) units apparently derived from *p*-coumaryl alcohol could be detected (1). This is consistent with the key role of the hydroxylase on the pathway toward coniferyl and sinapyl alcohols. C3H in *Arabidopsis* is now understood to operate on *p*-coumarate esters of shikimic or quinic acid, themselves produced by HCT (2). The difficulty in securing sufficient cell wall material from these stunted *ref8* mutants has thwarted the application of detailed structural studies of the resultant lignins.

Alfalfa Transgenics

One of our groups^g has successfully generated transgenic plants of the forage legume alfalfa (*Medicago sativa*) in which C3H levels have been reduced to as low as 5% of the wild-type level, in the absence of seriously impaired growth phenotypes (3). NMR spectra of various lignin fractions (e.g. Fig. 1) reveal both massive and subtle structural differences between the syringyl/guaiacyl lignins in normal wild-type alfalfa vs the *p*-hydroxyphenyl-rich lignins in the heavily C3H-down-regulated plants (4). They provide the first information regarding the incorporation profile for *p*-coumaryl alcohol into (*p*-hydroxyphenyl-rich) co-polymer lignins.

The anticipated effect of C3H-deficiency, an enhancement of the relative level of *p*-hydroxyphenyl (P) units in the lignin, is compellingly demonstrated in the aromatic profiles revealed by HSQC NMR

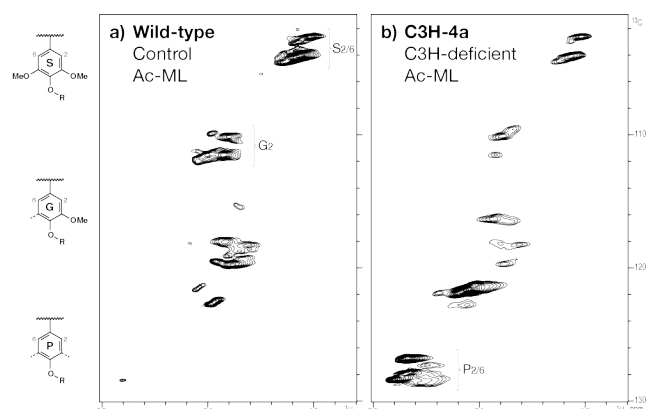


Fig. 1. Partial short-range ¹³C–¹H (HSQC) correlation spectra (aromatic regions only) of milled lignins (ML) isolated from **a)** the wild-type control and **b)** the most highly C3H-deficient line, C3H-4a. Traces of *p*-hydroxyphenyl (P) units are seen in the typically syringyl/guaiacyl (S/G) lignin in the wild-type alfalfa, whereas P-units entirely dominate the spectrum in the transgenic. Semi-quantitative volume integrals are given in Table 1.

spectra, Fig. 1. Wild-type plants have syringyl/ guaiacyl lignins with only low levels of P-units. Reduction of C3H depressed the synthesis of coniferyl and sinapyl alcohols although, as noted for other enzymes in the pathway, not in direct proportion to the enzyme expression level. The most severely down-regulated C3H-4a line (Fig. 1b) was G- and S-depleted and strikingly P-rich, about 65% P, Table 1.

The high-field HSQC spectra of the sidechain regions (not shown) are more revealing regarding the manner in which the monomeric units are assembled. Lignins are characterized by various types of interunit bonds, the most prominent being denoted **A-D**, **S**, and **X1** and **X7** in Table 1. This linkage-type distribution differs substantially between the wild-type and the C3H-depleted alfalfa lignins. The minor but important spirodienones **S**, resulting from β -1-coupling reactions, are absent in the C3H-deficient lignin, likely due to a simple chemical incompatibility with P-units. A multitude of dibenzodioxocins appear in the C3H-deficient lignins. The lower proportion of β -ether units **A** (~53-56% vs ~75-80% of the units quantified) is clearly a major reason for the lower thioacidolysis yields (on a lignin basis) for the C3H-deficient plants vs the wild-type (4). It also suggests that alkaline pulping efficiency will be lower, since pulping depends on ether cleavage reactions to depolymerize the lignin and render its fragments soluble in the pulping liquor. However, since lignin-polysaccharide cross-linking can occur via trapping of intermediate β -ether quinone methides during lignification, reducing the β -ether content may reduce lignin-polysaccharide cross-linking and produce cell walls that are more enzymatically degradable, as demonstrated for the C3H down-regulated lines via their improved digestibility in ruminant animals (3). Much of the decrease in β -ether **A** levels appears to be due to the two other major units, phenylcoumarans **B** and resinols **C**, each of which nearly doubles in relative proportion. The higher resinol concentration particularly suggests that more monomer-monomer coupling reactions are occurring during the lignification in the P-rich lignins. Although still quite low, the relative dibenzodioxocin **D** level is about double that in wild-type plants.

TABLE 1

NMR-derived *p*-Hydroxyphenyl:Guaiacyl:Syringyl (P:G:S) and Interunit Linkage Data for Stem Lignins from Control and C3H-Deficient Alfalfa

Sample	%P	%G	%S	%A	%B	%C	%D	%S	%X1	%X7
Control Ac-ML	0.8	58	41	75	9	9	1.1	0.6	4.8	0.5
Control Ac-AL	0.7	61	39	80	8	7	0.6	0.2	3.7	0.4
Control Ac-EL	0.8	58	41	77	8	8	0.7	0.6	4.7	0.6
C3H' Ac-ML	65	17	18	56	18	16	2.6	-	2.9	4.6
C3H' Ac-AL	68	17	15	56	16	14	2.1	-	5.3	6.0
C3H' Ac-EL	66	16	18	53	17	16	1.8	-	5.6	6.6

Fractions: ML = dioxane:water-soluble milled lignin, AL = acidolysis lignin (from the ML residue), EL = enzyme-digested cell wall; Ac- indicates acetylated samples. C3H-deficient transgenic has 5% residual C3H levels. **P** = *p*-hydroxyphenyl; **G** = guaiacyl; **S** = syringyl; **A** = β -O-4 (β -aryl ether); **B** = β -5 (phenylcoumaran); **C** = β - β (resinol); **D** = dibenzodioxocin; **S** = β -1 (spirodienone); **X1** = cinnamyl alcohol endgroup; **X7** = arylglycerol endgroup.

Poplar and Pine Transgenics

Two groups^{e,h} have collaboratively generated C3H-deficient poplar, yet another^f has HCT-downregulated *P. radiata* tracheid cultures, and one group^g now also has HCT-downregulated alfalfa. As this abstract is being written, only cursory structural information is available on these materials, but analysis is expected to be completed before the meeting and will be reported.

References

1. Franke, R., Humphreys, J. M., Hemm, M. R., Denault, J. W., Ruegger, M. O., Cusumano, J. C., and Chapple, C. (2002) *Plant J.* **30**(1), 33-45
2. Schoch, G., Goepfert, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., and Werck-Reichhart, D. (2001) *J. Biol. Chem.* **276**(39), 36566-36574
3. Reddy, M. S. S., Chen, F., Shadle, G. L., Jackson, L., Aljoe, H., and Dixon, R. A. (2005) *Proc. Nat. Acad. Sci.* **102**(46), 16573-16578
4. Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P. F., Marita, J. M., Ralph, S. A., Reddy, M. S. S., Chen, F., and Dixon, R. A. (2006) *J. Biol. Chem.* **281**(13), 8843-8853

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