CONTROL OF CELL WALL COMPONENT BIOSYNTHESIS IN WOOD FORMATION

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ABSTRACT

This paper summarizes our previous and current research on genetic engineering of lignin biosynthesis for the purposes of improving wood pulping and bleaching efficiency. For these purposes, our targets were to produce transgenic trees with low content of lignin that is also chemically reactive (high lignin S/G ratio). Using aspen as a model species, we have characterized the biochemical functions of various genes and the kinetic properties of these gene products involved in monolignol biosynthetic pathway. The results of these characterizations proved strong evidence for a principle phenolic flux leading to the formation of monolignols. Biochemical evidence further demonstrated that, in this principle flux, 4CL could be the enzyme limiting total lignin accumulation, whereas CAld5H might control the lignin S/G ratio. These propositions were fully supported by the *in vivo* functions of these enzymes. Transgenic trees with inhibited 4CL enzyme activity exhibited 5 to 45% reduction in lignin contents. The chemical structure of the resulting lignin remained essentially unchanged. More importantly, the lignin reduction was compensated for by a concomitant increase in cellulose content. When antisense *4CL* and sense *CAld5H* genes were simultaneously transferred into aspen via *Agrobacterium*, transgenic trees expressing each one and both of the transgenes were produced. Lignin reductions up to 55% were achieved in antisense *4CL* plants and up to 3-fold S/G increases were observed in sense *CAld5H* plants. These effects were independent but additive, with plants expressing both transgenes having less lignin and higher S/G ratio. Consistent with our previous results, lignin reduction has always resulted in an increase in cellulose content. These transgenics could be potentially valuable for pulp production. But more importantly, these benchmark transgenics are rich sources of information for functional genomics and metabolic engineering, allowing the generation of the ultimate raw materials for wood pulp production.

RESULTS AND DISCUSSION

For more than 50 years our thinking of syringyl monolignol biosynthesis in angiosperms has adhered to the paradigm that a ferulate pathway (Fig. 1) from caffeate to sinapate via ferulate and 5-hydroxyferulate would lead to such a biosynthesis.[1-3] Based on HPLC/MS characterization of products from reactions of microsomal proteins from lignifying stem xylem of sweetgum (*Liquidambar styraciflua*) with a mixture of four potential 5-hydroxylation substrates, ferulate, feruloyl-CoA, coniferaldehyde and coniferyl alcohol, Osakabe *et al.*[4] discovered that 5-hydroxyferulate was not synthesized. Instead, the exclusive product from this mixed substrate reaction was 5-hydroxyconiferaldehyde, demonstrating for the first time that a coniferaldehyde 5-hydroxylase (CAld5H) is involved in monolignol biosynthesis, and F5H may not be.[4] Subsequently, *CAld5H* cDNAs were cloned from aspen and sweetgum.[4] When coniferaldehyde was incubated with a mixture of CAld5H-containing yeast P450 and *E. coli*- expressed caffeate *O*-methyltransferase (COMT), it was converted to sinapaldehyde via 5-hydroxyconiferaldehyde.[4] Thus, CAld5H catalyzes 5-hydroxylation of coniferaldehyde into 5-hydroxyconiferaldehyde, which in turn is methylated by COMT to sinapaldehyde, supporting the idea of a hydroxylation/methylation flux *in vivo* from guaiacyl to syringyl monolignol biosynthesis via coniferaldehyde (Fig. 1). Based on HPLC/MS characterization of the kinetic properties of purified recombinant aspen COMT, Li *et al.*[5] demonstrated that, indeed, COMT is a 5-hydroxyconiferaldehyde *O*-methyltransferase (AldOMT) that catalyzes methylation of 5-hydroxyconiferaldehyde (K_m = 2.6 μM) with some affinity for caffeate (K_m = 75.1 μM) and 5-hydroxyferulate (K_m = 15.0 μM).

However, when a mixture of 5-methylation substrates caffeate, 5-hydroxyferulate and 5-hydroxyconiferaldehyde was incubated with recombinant COMT (now designated as AldOMT) or soluble proteins from stem xylem, a complete inhibition of caffeate and 5-hydroxyferulate methylation was observed, while the conversion of 5-hydroxyconiferaldehyde into sinapaldehyde (Fig. 1) was conserved.[5] Enzyme inhibition kinetics further showed that 5-hydroxyconiferaldehyde is a competitive inhibitor of AldOMT-catalyzed methylation of both 5-hydroxyferulate and caffeate with K_i values of 0.26 and 2.1 μM, respectively, but 5-hydroxyferulate and caffeate are not effective inhibitors of 5-hydroxyconiferaldehyde methylation.[5] Thus, the presence of CAld5H/AldOMT-mediated coniferaldehyde 5-hydroxylation/methylation eliminates the pathway from caffeate to sinapate via ferulate and 5-hydroxyferulate (Fig. 1), and CAld5H/AldOMT diverts the guaiacyl pathway from coniferaldehyde to sinapaldehyde via 5-hydroxyconiferaldehyde to initiate syringyl monolignol biosynthesis (Fig. 1).

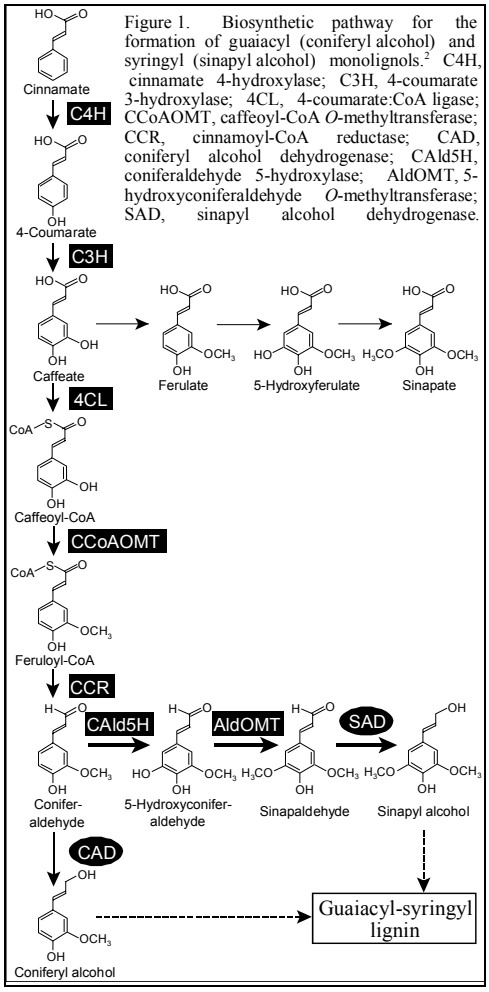

Figure 1. Biosynthetic pathway for the formation of guaiacyl (coniferyl alcohol) and syringyl (sinapyl alcohol) monolignols.[2] C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; 4CL, 4-coumarate:CoA ligase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, coniferyl alcohol dehydrogenase; CAld5H, coniferaldehyde 5-hydroxylase; AldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; SAD, sinapyl alcohol dehydrogenase.

The CAld5H/AldOMT pathway together with the long-thought CAD function with sinapaldehyde was once believed to lead to the biosynthesis of syringyl monolignol. However, HPLC/MS-based enzyme functional analyses of aspen xylem protein and *E. coli*-expressed recombinant aspen CAD protein demonstrated that CAD is in fact coniferaldehyde- or guaiacyl-specific.[6] This strongly suggests that a discrete sinapyl alcohol dehydrogenase (SAD) is needed for metabolizing the CAld5H/AldOMT product, sinapaldehyde, into sinapyl alcohol, the syringyl monolignol. This discovery led to the isolation of an SAD cDNA from aspen developing xylem.[6] Like the CAld5H/AldOMT-mediated initiation of the syringyl pathway, SAD protein is widely distributed in angiosperms,[5] but SAD as well as CAld5H and AldOMT proteins and their functions are absent from gymnosperms.[6] These results challenge the traditional model of monolignol biosynthesis and suggest that CAD mediates the reduction of coniferaldehyde into guaiacyl monolignol and that SAD along with CAld5H/AldOMT controls the biosynthesis and utilization of sinapaldehyde for syringyl monolignol.

Biochemical evidence further demonstrated that, in this principle flux, 4CL could be limiting total lignin accumulation.[7] 4CL, an enzyme upstream of coniferaldehdye (Fig. 1), has been demonstrated to limit lignin accumulation in various plants. Transgenic aspen trees with downregulated lignin-specific 4CL, Pt4CL1,[7] exhibited up to a 45% reduction of lignin, but this did not alter lignin structure with respect to S/G ratio, as revealed by lignin thioacidolysis.[7] Two-dimensional HSQC NMR further confirmed that the common lignin structural units are all similarly represented in wild type and lignin-reduced transgenic trees.[7] Thus, these data provide strong evidence for the absence of any significant branch pathways at caffeate, the preferred 4CL substrate,[8] that would otherwise divert caffeate metabolism away from the principal phenolic flux (Fig. 1) to result an abnormal type of lignin. We proposed that, with respect to this principal flux, the result of 4CL downregulation is simply the attenuation of metabolite pools downstream of caffeate, limiting the availability of the normal precursors, the monolignols, for lignin polymerization.

Table 1. Chemical compositions in stem wood of control and transgenic aspen

Plant	Control	21	22	23	25	36	32	84	96	94	102	72	74	141	143
Gene integrated															
-4CL[a]		*-4CL*	*-4CL*	*-4CL*	*-4CL*	*-4CL*						-4CL	-4CL	-4CL	-4CL
+CAld5H[b]							*+CAld5H*	*+CAld5H*	*+CAld5H*	*+CAld5H*	*+CAld5H*	+CAld5H	+CAld5H	+CAld5H	+CAld5H
Lignin content (%)[c]	22.2 ± 0.8	16.0 ± 0.6	15.3 ± 0.4	14.4 ± 0.5	13.1 ± 0.3	14.9 ± 0.2	22.4 ± 0.5	21.6 ± 0.4	21.1 ± 0.4	20.7± 0.6	19.7± 0.4	13.7± 0.4	12.4± 0.5	10.7± 0.4	13.2± 0.3
Lignin S/G ratio	2.2	2.1	2.0	2.2	2.3	2.1	4.8	4.0	5.5	4.9	3.0	3.6	3.4	2.7	3.3
Cellulose content (%)[d]	41.4±0.4	43.1±0.3	ND	44.8±0.4	47.3±0.5	ND	40.0±0.3	42.6±0.2	44.7±0.1	43.4±0.2	44.3±0.1	49.2±0.3	50.9±0.1	53.3±0.2	ND
Xylan content (%)[e]	15.8±0.2	16.8±0.3	ND	16.1±0.3	16.9±0.4	ND	16.5±0.4	15.3±0.1	15.7±0.5	15.6±0.5	15.2±0.1	15.3±0.3	14.6±0.2	15.4±0.4	ND
Cellulose/lignin ratio	1.9	2.7	ND	3.1	3.6	ND	1.8	1.9	2.1	2.0	2.2	3.6	4.1	5.0	ND

Values are means ± SE of two to three assays of different samples from each line. [a](*-4CL*) and [b](*+CAld5H*) denote antisense *4CL* and sense *CAld5H* transgenes, respectively. [c]Lignin, [d]cellulose, and [e]xylan contents are % of dry wood weight. ND: not determined.

When antisense *4CL* and sense *CAld5H* genes were simultaneously transferred into aspen via *Agrobacterium*, phenotypically normal transgenic trees expressing each one and both of the transgenes were produced.[9] Forty transgenic aspen lines were obtained, of which 37, 40, and 23% harbored antisense *Pt4CL*, sense *LsCAld5H* and antisense *Pt4CL* + sense *LsCAld5H* gene constructs, respectively, as confirmed by genomic PCR. From each of these three transgenic groups grown in a greenhouse, several trees were randomly selected and harvested at the age of 10 months during the growing season for various characterizations. 4CL protein levels were drastically reduced in lines harboring only antisense *Pt4CL* transgene, leading to a 70-90% reduction in xylem 4CL enzyme activity, and a 30-40% reduction in stem lignin (Table 1). No significant effect on the lignin S/G ratio was found (Table 1). Overexpressing the *LsCAld5H* gene alone drastically elevated the xylem CAld5H protein levels, giving rise to a 2.2-2.8-fold increase in xylem CAld5H enzyme activity. As a result, these transgenics exhibited up to a remarkable 2.5-fold increase in the S/G ratio as compared to the control (Table 1). The single *CAld5H* gene effect had no influence on total lignin accumulation in transgenic trees (Table 1).

However, the single-gene effects became additive in transgenics harboring both antisense *Pt4CL* and sense *LsCAld5H* genes. Alterations of 4CL and CAld5H protein levels in these trees were consistent with changes of the corresponding enzyme activities; 80-90% reduction in 4CL and 60 to 110% increase in CAld5H. This combinatorial gene manipulation had led to a 38-52% reduction in stem lignin and a 22-64% increase in the lignin S/G ratio (Table 1).

Transgenic trees with reduced lignin exhibited an increase in cellulose content, and an up to a remarkable 30% increase was observed in antisense-*Pt4CL*/sense-*LsCAld5H* transgenic line 141 due to a 52% lignin reduction (Table 1). Consistent with the observation reported by Hu *et al*.,[7] the increased cellulose content together with reduced lignin quantity resulted in a cellulose:lignin ratio of 3 to 5 in the transgenic lines, as opposed to 2.2 in the control (Table 1). The relative abundance of the major hemicellulose component xylan was essentially unaffected in all transgenic lines, confirming our previous results.[7]

CONCLUSION

Lignin reductions in trees can be achieved by antisense *4CL* technology and overexpression of sense *CAld5H* would result in S/G increases. These effects were independent but additive, with plants expressing both transgenes having less lignin, higher S/G ratio and more cellulose. These transgenics would be potentially valuable lignocellulosic substrates for woodpulp production. These transgenics may not be the ultimate lignocellulosics for ethanol. But they are benchmark transgenics and are rich sources of information for understanding cell wall biosynthesis and thus for further metabolic engineering, allowing the generation of the ultimate raw materials for woodpulp production.

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