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Biosynthesis of monolignols. Genomic and reverse genetic approaches

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Abstract

The biosynthesis of monolignols is one of the most studied pathways of plant natural product biosynthesis. However, the pathway has recently undergone considerable revision, and it would appear that our understanding of the exact routes for synthesis of the building blocks of lignin and lignans is still not fully understood. Early studies of *in vitro* enzyme specificity failed to appreciate the catalytic promiscuity of some of the enzymes of the monolignol pathway, and the evolving model of a metabolic grid for monolignol biosynthesis may fail to appreciate the possible extent of metabolic channeling within the pathway. New approaches to the study of monolignol biosynthesis include genomics, advanced cellular imaging techniques, and transgenic manipulation. This article summarizes the use of these approaches to gain a better understanding of the operation of a complex metabolic pathway.

Introduction

Lignin is a complex polymer of hydroxylated and methoxylated phenylpropane units, linked via oxidative coupling (Boudet et al., 1995), and serves as a major structural component of secondarily thickened plant cell walls. It imparts mechanical strength to plant stems and trunks, and hydrophobicity to water-conducting vascular elements. Because of the negative effects of lignin on paper pulping and forage quality, there is considerable interest in genetic manipulation to alter the quantity and/or quality of the lignin polymer (Whetten and Sederoff, 1991; Boudet and Grima-Pettenati, 1996; Dixon et al., 1996; O'Connell et al., 1998). Many lignans originate from the monolignol coniferyl alcohol, and genetic modification of lignans is also of interest in relation to potential human health benefits, since grain seed lignans such as secoisolariciresinol and matairesinol are converted by intestinal microflora to enterolactone and enterodiol, estrogenic compounds with potentially positive effects in relation to cancer and cardiovascular disease (Mazur and Adlercreutz, 1998). These approaches require an understanding of the various enzymatic and regulatory processes involved in monolignol biosynthesis and lignin polymerization and deposition.

Dicotyledonous angiosperm lignins contain two major monomer species, termed guaiacyl (G) and syringyl (S) units. G units are characterized by a single methoxyl substituent on the aromatic ring and arise from coniferyl alcohol, whereas S units are di-methoxylated and arise from sinapyl alcohol (Figure 1). Lignin from monocotyledonous plants also contains a significant proportion of H units derived from coumaryl alcohol. The monomeric units in lignin are joined through at least five different types of linkages (Davin and Lewis, 1992), and polymerization proceeds via free radical reactions initiated by the enzymes peroxidase and laccase (Sato et al., 1993; Bao et al., 1993). The mechanisms that determine the relative proportions of the linkage types in a particular lignin polymer are currently unknown. However, lignin composition is developmentally programmed, both temporally and spatially (Terashima et al., 1993).

Although the exact mechanisms underlying the initiation, growth and termination of the lignin polymer remain to be determined, much more is known concerning the biosynthetic pathways leading to the individual monolignols. Although studies on monolignol biosynthesis have a long history (Lewis et al., 1999), the past two years have seen major re-evaluations of the pathway (Dixon et al., 2001; Humphreys and

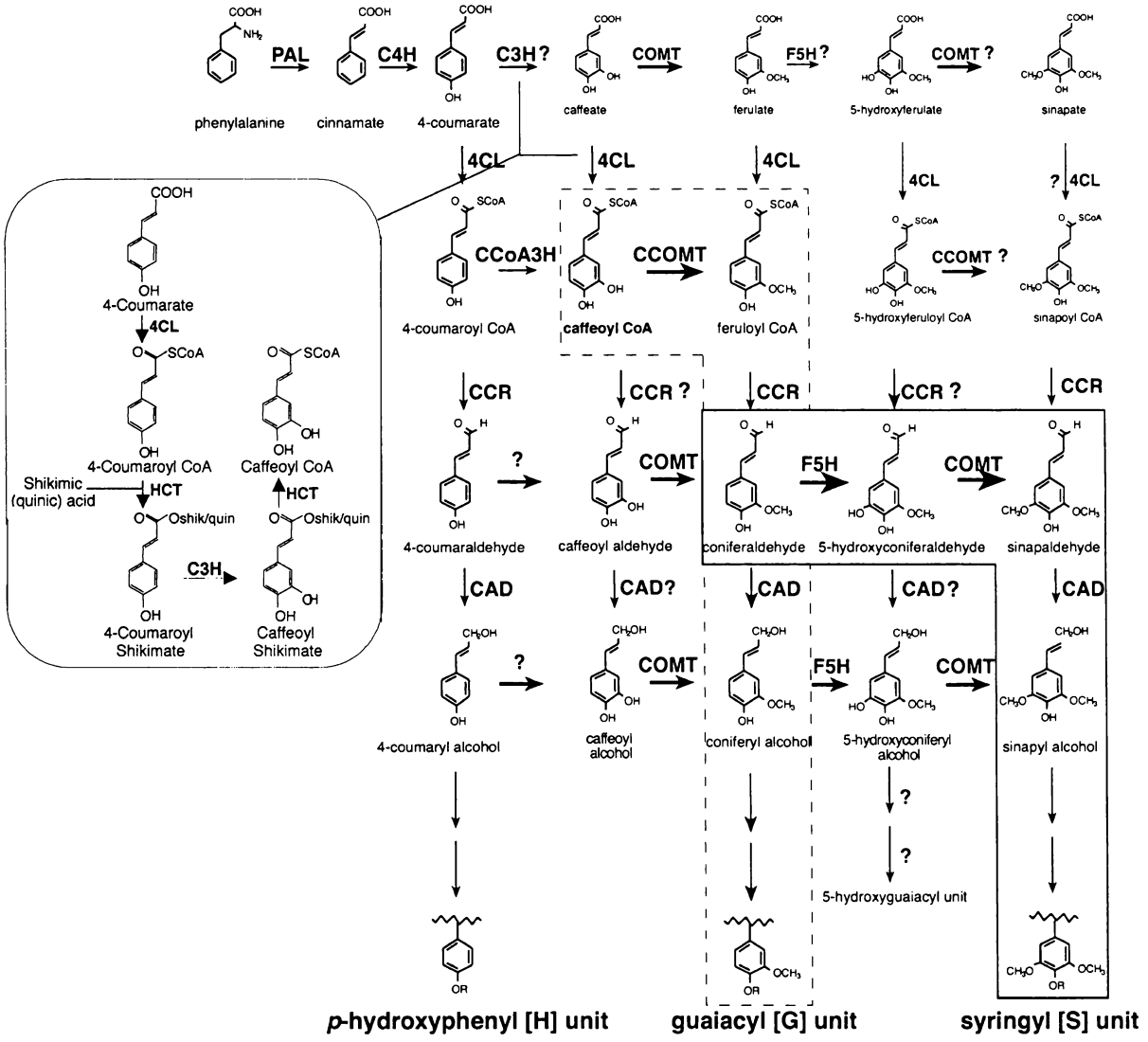

Figure 1. Scheme showing the proposed pathways leading to the biosynthesis of guaicayl and syringyl monolignols. This scheme incorporates data from *in vitro* enzymatic studies in several different plant species, and may not represent the actual *in vivo* pathway in any specific species. See text for details of the various enzymes.

Chapple, 2002), in part based on the results of reverse genetic approaches aimed at modification of lignin content and composition in model species. This chapter reviews the development of our ideas on biosynthetic pathways leading to monolignols, and assesses the impact of new genomics technologies and genetic manipulation approaches on our understanding of lignin biosynthesis.

The monolignol pathway – Evolution of ideas

Until recently, our understanding of the ring substitution reactions of monolignol biosynthesis had been largely based on substrate specificity studies *in vitro* with available assumed substrates. Such an approach has formed the basis of much of the discipline of biochemistry, where reactions shown to occur *in vitro* are generally assumed to also function *in vivo*. However, in the case of monolignol biosynthesis, it is now clear that some of the enzymes are relatively promiscuous, and some substrates initially believed to be of

physiological significance may not be actual pathway intermediates *in vivo*.

Figure 1 presents a diagrammatic summary of the monolignol pathway as currently perceived. It was initially thought that the successive ring hydroxylations and *O*-methylations in monolignol biosynthesis took place at the level of hydroxycinnamic acids (Neish, 1968). For example, caffeic acid *O*-methyltransferase (COMT) was first shown to convert caffeic acid to ferulic acid, and was later shown also to convert 5-hydroxyferulic acid to sinapic acid (Davin and Lewis, 1992). Two challenges to this view of monolignol formation came with the realizations that, firstly, there appeared to be an alternative pathway for methylation at the coenzyme A ester level involving caffeoyl CoA *O*-methyltransferase (Ye et al., 1994). This enzyme is more closely related to mammalian catechol OMT than to plant COMT at the DNA sequence level (Joshi and Chiang, 1998). Second, the enzyme known as ferulate 5-hydroxylase (F5H) has only low activity with ferulic acid as substrate, a finding that questioned the operation of the pathway at the free acid level. It was subsequently demonstrated that F5H was much more active with coniferaldehyde as substrate, and that 5-hydroxyconiferaldehyde was a preferred substrate for COMT (Humphreys et al., 1999; Osakabe et al., 1999). Inhibition of COMT-mediated methylation of caffeic acid *in vitro* by 5-hydroxyconiferaldehyde could, if operational *in vivo*, essentially prevent flux from caffeic acid to ferulic acid from occurring (Li et al., 2000). These findings led to the suggestion that hydroxylation and *O*-methylation in monolignol biosynthesis occur at the level of the coenzyme A ester (for 3-*O*-methylation) or aldehyde (for 5-*O*-methylation). This was not the end of the story, however. *In vivo* labeling experiments in *Magnolia kobus* indicated that S lignin can be derived from coniferyl alcohol. In these studies, side-chain labeled coniferyl alcohol was incorporated into S lignin with complete retention of label, indicating direct incorporation (Matsui et al., 1994; Chen et al., 1999). Consistent with a pathway for hydroxylation and methylation at the alcohol level, COMT from alfalfa has a significantly higher affinity for 5-hydroxyconiferyl alcohol than for caffeic acid (Parvathi et al., 2001). Finally, recombinant alfalfa COMT can also efficiently catalyze the 3-*O*-methylation of caffeyl aldehyde and alcohol, suggesting that COMT might also be involved in the 3-*O*-methylation of monolignol precursors at the aldehyde or alcohol levels (Parvathi et al., 2001).

After many years of frustrated attempts to discover an enzyme that specifically hydroxylated 4-coumarate to yield caffeate (coumarate 3-hydroxylase) and whose properties suggested an involvement in lignification (Bolwell and Butt, 1983), it has recently become clear that the enzyme that carries out this function acts not on free 4-coumarate but on a carboxylate ester that, *in vitro*, may be the ethyl, shikmate or quinate ester (Schoch et al., 2001; Franke et al., 2002; Franke et al., 2002). This enzyme is a cytochrome P450 (CYP98A3), and mutant Arabidopsis plants in which it is not expressed accumulate a novel lignin consisting primarily of H units, indicating that this enzyme is indeed involved in the 3-hydroxylation reaction of lignin biosynthesis (Franke et al., 2002). A major question now is whether other reactions in the monolignol pathway might also take place with previously unsuspected esterified substrates; this possibility is raised by the results of certain genetic modification experiments (see below).

The operation of the "shikimate ester shunt" drew attention to the possible role of the quinate/shikimate hydroxycinnamoyl transferase (HCT), an enzyme that had previously been related to biosynthesis of chlorogenic acid (3-*O*-caffeoyl quinate) (Ulbrich and Zenk, 1979), in monolignol biosynthesis. This enzyme is reversible, and would have to function in both forward and reverse directions in the scheme in Figure 1. The gene encoding this enzyme has recently been cloned from tobacco (Hoffmann et al., 2003).

Reduction of hydroxycinnamic acids to their corresponding alcohols is initiated by formation of coenzyme A thioesters that are reduced successively to the corresponding aldehydes by cinnamoyl CoA reductase(s) (CCR) and cinnamyl alcohol dehydrogenases(s) (CAD). Hydroxycinnamate: CoA ligases (4CL) have been characterized at the molecular level from many plant species. In general, these enzymes exhibit highest activity against coumaric, caffeic and ferulic acids, with little activity against cinnamic and sinapic acids (Ehlting et al., 1999). The apparent lack of activity of 4CL against sinapic acid in several plants calls into question the role of sinapate as a lignin precursor, consistent with the pathways proposed in Figure 1. A recent study that combined analysis of 4CL specificity with *in vivo* labeling with sinapate has confirmed that, in Arabidopsis and magnolia, sinapate is not an intermediate in S lignin biosynthesis (Yamauchi et al., 2003). However, in oleander and robinia, 4CL was active with sinapate and labeled sinapate was incorporated into lignin. Thus, S lignin biosynthesis in

angiosperms can occur by multiple pathways that are species-specific.

Additional enzymes may catalyze the methylation reactions shown in Figure 1. For example, young alfalfa internodes contain an OMT (COMT II), with preference for caffeic acid compared to 5-hydroxyferulic acid. This enzyme can be separated from the previously characterized COMT (COMT I) by anion exchange and hydrophobic interaction chromatography (Inoue et al., 2000). COMT II differs from COMT I in its native Mr, pH optimum, and its very low Km for caffeic acid. COMT II may therefore be a true caffeic acid OMT (although the enzyme is also active with coenzyme A esters and some flavonoids), and may be involved in formation of ferulic acid in the earliest stages of lignification in young tissues. A similar "early" form of COMT has been described in wheat seedlings (Lam et al., 1996). Another interesting OMT potentially involved in lignification has been identified in loblolly pine (Li et al., 1997). Called AEOMT (acids and esters OMT), this enzyme has approximately equal preference for caffeic acid and caffeoyl CoA. To date, a potential function for this enzyme in lignification has only been inferred from its *in vitro* biochemical activities.

Regulatory genes for monolignol biosynthesis

The concentrations of the inter- and extra-cellular proteins involved in monolignol biosynthesis and polymerization are controlled, in large part, by transcriptional events. Although the transcriptional control of monolignol biosynthetic genes in relation to vascular development is not well understood, several transcription factors have been shown to affect lignification. These have been identified by two distinct approaches: analysis of factors with binding affinity for *cis*-elements in the promoters of monolignol biosynthetic genes, and mutational analysis.

The *Antirrhinum* MYB transcription factors AmMYB308 and AmMYB330, when over-expressed in transgenic tobacco, repress lignin biosynthesis (Tamagnone et al., 1998). Expression of AmMYB 308 strongly reduced steady state transcript levels of 4CL and CAD, with a smaller effect on cinnamate 4-hydroxylase (C4H) and no effect on phenylalanine ammonialyase (PAL) transcripts. *In vitro*, AmMYB308 was a weak transcriptional activator of the 4CL promoter, leading to the suggestion that the repressive effect of over-expression was due to competition for the natural MYB regulator of 4CL expression (Tamagnone et al., 1998). PAL expression is up-regulated by expression of a different MYB protein, AtMYM305, in tobacco (Urao et al., 1993). Many phenylpropanoid pathway genes contain binding sites for MYB family transcription factors in their promoters (Martin and Paz-Ares, 1997), and some *MYB* genes are highly expressed in stem tissue undergoing vascular differentiation (Martin and Paz-Ares, 1997). These observations suggest novel approaches for modification of lignin content and composition by altering expression of endogenous MYB transcription factors or by interfering with endogenous MYB protein function by expression of foreign *MYB* genes.

Genomics approaches to lignin biosynthesis in *Medicago truncatula*

Medicago truncatula (also known as barrel medic because of the shape of its seed pods) is a forage legume commonly grown in Australia. It originates from Mediterranean regions, and has recently been introduced as a warm season annual legume to the Gulf Coast States in the US. *M. truncatula* is very closely related to the world's major forage legume, alfalfa (*Medicago sativa*). However, whereas alfalfa has a complex genome consisting of four copies of each of its eight chromosomes and is an outcrossing plant, *M. truncatula* has a simple diploid genome and can be self-pollinated, facilitating genetic analysis.

M. truncatula has been chosen as a model species for genomic studies in view of its small genome, fast generation time and genetic transformation efficiency (Cook, 1999). Genes from *M. truncatula* share very high sequence identity to their counterparts from alfalfa, and also appear to be arranged in a similar order on the chromosomes, making *M. truncatula* an excellent model for understanding the molecular biology of alfalfa. We have utilized *M. truncatula* as a model species for a genomics-based approach to understanding monolignol biosynthesis. The rationale for using this species is two-fold. First, from a practical viewpoint, the other genomically tractable model dicot, Arabidopsis, is a small plant with a rosette habit that is less than optimal for studies on lignification in vegetative stem tissue. Secondly, lignin modification is an important trait in *Medicago* species in relation to its impact on forage digestibility.

The development of rapid expressed sequence tag (EST) and genomic sequencing technologies has al-

lowed an unprecedented increase in our understanding of the complexity of gene families in plants. It is now possible, for several model and agronomically important plants, to view and analyze sequences of all gene family members, and compute their expression patterns *in silico*, by simple search and query commands with various Plant Gene Index databases, such as those available at the TIGR website (http://www.tigr.org/tdb/tgi.shtml) (Quackenbush et al., 2000). The *Medicago* gene index at the National Center for Genome Resources (Bell et al., 2001) and the TIGR *Medicago* gene index, provide information on over 190,000 ESTs from *M. truncatula*, and a whole genome sequence for *M. truncatula* was recently announced (Trends in Plant Science 7, 101, 2002). An important feature of the *M. truncatula* EST data is that nearly 40 different cDNA libraries, representing a range of tissues and biological conditions, have been sequenced, greatly facilitating *in silico* analysis of gene expression patterns.

Comparative genomics of the lignin biosynthetic pathway

Table 1 summarizes the apparent numbers of gene family members for various genes potentially involved in the biosynthesis of monolignols in *Medicago truncatula*, Arabidopsis, rice and maize. The numbers refer to tentative consensus sequences (TCs) that represent EST contigs derived from clustering of the EST sequences. Every TC annotated in the database as representing a specific gene product has been counted as such. Singletons (sequences that occur only once in the database and do not show overlap with other EST sequences) are also included in the analysis. With over 200,000 ESTs now sequenced in *M. truncatula*, the data in Table 1 probably represent a fairly accurate picture of gene family complexity.

Two important conclusions can be made from the data in Table 1. First, in all four species, many of the genes exist as large gene families. In the cases of *4CL*, *CCR*, and *CAD*, these may have ten or more members. Second, the levels of complexity differ between the different species e.g. a single *4CL* gene in rice, 10 or more in Arabidopsis and *M. truncatula*. In spite of extensive EST sequencing, some genes that must exist have yet to be represented in the EST databases, such as C4H in maize. This may be a problem of selectivity of sequenced libraries, low transcript abundance, or poor annotation.

As a note of caution, the EST counting approach annotates genes based solely on sequence similarity, and does not infer functional identification of a particular gene product. Thus, some of the genes annotated as encoding a particular enzyme may in fact encode related enzymes with different but related functions. Only *in vitro* expression and assay of the individual forms can distinguish between these possibilities. Furthermore, there is emerging evidence to indicate that some enzymes of natural product biosynthesis, including "monolignol" enzymes such as COMT, have relatively promiscuous substrate specificities (Maury et al., 1999), and could theoretically be involved in multiple pathways. Reverse genetic approaches will be required to resolve such issues.

To date, most transgenic approaches to lignin modification have not taken into account the potential complexity of the gene families being targeted. It is thus possible that closely related genes in addition to the target gene have been down-regulated, or less related genes with the same function have escaped down-regulation. Definitive information as to why many of the gene families in Table 1 are so complex, and what the functions of the specific gene family members are, will require specific down-regulation of the individual gene forms. This has been problematical in the past owing to the often very high DNA sequence conservation between family members. Because of this, use of antisense or gene silencing with large sequence fragments would result in down-regulation of several or maybe all the genes. Recent advances in plant gene silencing technology based on an understanding of RNA-interference (RNAi) (Wesley et al., 2001) should now facilitate the molecular dissection of the functions of individual members of the monolignol pathway gene families, with the proviso that overlapping specificities and gene redundancy might complicate the picture. This information may allow for more precise engineering of lignin and lignan biosynthesis. It is intriguing to consider the possibility that some gene family members may be specific for lignin biosynthesis, with others involved in parallel reactions in lignan formation.

Lignin pathway gene complexity in *Medicago truncatula*

Phenylalanine ammonia-lyase (PAL). PAL catalyzes the entry point reaction into phenylpropanoid biosynthesis and as such is expected to be an early regu-

Table 1. Gene family members involved in the biosynthesis of monolignols in *Medicago truncatula*

Enzyme name	Tentative consensuses (TCs) or singletons in TIGR databases					
	M. truncatula	Soybean	Tomato	Arabidopsis	Rice	Maize
Phenylalanine ammonia-lyase (PAL)						
	TC60344	TC120178	TC98559	TC149606	TC100267	TC155705
	TC64086	TC120179	TC98560	TC149607	TC104767	TC159236
	TC65562	TC120180	TC98561	TC149868	TC104764	TC159237
	TC68095	TC126818	TC98669	TC149869	TC104765	TC159238
	TC68096		TC105208	TC158072	TC104766	TC159239
	TC68097		TC107313	T45207	TC104768	TC160541
	TC68378		TC110806		AW155403	TC160542
	TC68379		TC111296		AW155576	TC160543
	TC68380		AW035278		D49142	
			BI203204		AU068510	
			BG735223		BE040736	
Cinnamate 4-hydroxylase (C4H)						
	TC68098	TC132363	TC110509	TC150023	AU058037	
	TC68099	TC132364				
4-Coumarate:coenzyme A ligase (4CL)						
	TC60026	TC120553	TC98405	TC150930	TC97362	TC151583
	TC65453	TC133962	TC99481	TC150931	TC99641	TC151664
	TC65681	TC120554	TC100408	TC151848	TC100881	TC161152
	TC66680	TC120557	TC101174	TC153791	TC103416	TC163817
	TC69081	TC124508	TC103426	TC163721	TC105867	TC167404
	TC69772	TC125645	TC104412	TC167534	TC106460	
	TC70400	TC128381	TC106474	NP281186	TC106531	
	TC71682	TC131261	TC106804		TC108475	
	TC72216	TC133929	TC112965		TC108664	
	TC75402	TC136358	AW031547		TC110024	
		TC136890	AW034240		NP002407	
			AW039905		NP302638	
			AW616655		NP409757	
			AW625022		AW155336	
			BE449653			
Hydroxycinnamoyl transferase (HCT)						
	TC59732	TC133435	TC100265	TC156505	TC104503	TC150657
		TC133436	TC100669	TC161029	TC105232	TC153392
		TC133437		TC161691	TC107940	
					NP451642	
Coumarate 3-hydroxylase (C3H)						
	TC60163	TC133516	TC102069	TC149793		
			TC107838			
			TC108065			
Caffeic acid *O*-methyl-transferase (COMT)						
	TC51781	TC133647	TC101479	TC152369	TC97209	TC149270
	TC59577	TC133648	TC101929	TC155499		TC149271
	TC59579		TC109048	TC156743		TC151662
	TC59990			TC162925		
	TC67955			TC162926		
	TC67956			NP236939		
	TC69180					
	TC74636					

Table 1. Continued

Enzyme name	Tentative consensuses (TCs) or singletons in TIGR databases					
	M. truncatula	Soybean	Tomato	Arabidopsis	Rice	Maize
Caffeoyl coenzyme A *O*-methyl-transferase (CCoAOMT)						
	TC61903	TC123959	TC98418	TC149810	TC98369	TC159998
	TC61904	TC126458	TC98738	TC153568	TC104956	TC160367
	TC68371	TC127146	TC98739	TC153569	NP001843	TC152158
	TC68997	TC132670	TC100264	TC170490		
		TC132671	TC102387	AA394533		
		TC132673	AI776146			
		TC132674	AW219626			
		TC132745				
		TC132746				
		TC132748				
Ferulate 5-hydroxylase (F5H)						
	TC65237	TC122880	TC102880	TC150466	TC108119	
	TC68915		TC106767	NO281450		
Cinnamoyl coenzyme A reductase (CCR)						
	TC59785	TC121013	TC100442	TC154177	TC97148	TC149677
	TC70266	TC121015	TC102105	TC155063	TC97149	TC149679
	TC68330	TC124904	TC107062	TC161141	TC97789	TC150846
	TC60893	TC128675	TC107476	TC161670	TC98316	TC152275
	TC60894	TC129395	TC109165	TC166923	TC99014	TC154542
	TC68604	TC132311		TC157466	TC100822	TC157145
		TC132313		TC161648	TC100974	TC166720
		TC134196		TC161649	TC101444	NP003454
		TC134197		TC167535	TC106057	NP003455
		TC139658		TC167541	TC107202	
		TC139724		BE522400	TC107430	
					TC107510	
					AU064260	
					AW155595	
					C73909	
					BI306691	
					AU174135	
					D48135	
Cinnamyl alcohol dehydrogenase (CAD)						
	TC61379	TC125122	TC99774	TC150052	TC105400	TC160262
	TC61380	TC132545	TC100576	TC150367	TC108577	TC162614
	TC62212	TC132547	TC102029	TC150431	TC105215	TC155193
	TC62213	TC132548	TC105106	TC154254	TC106887	TC150069
	TC67973	TC132604	TC106936	TC161262	TC107624	TC157053
	TC68444	TC132605	AW037980	TC161322	TC100053	
	TC72882	TC132606		TC168050	TC107701	
	AW696839	TC133407		TC159031	TC112544	
	AW559294			TC165297	TC99454	
				NP040876		
				NP040879		
				NP307424		

latory control point for monolignol biosynthesis. The *in silico* expression pattern of the nine *M. truncatula PAL* or *PAL*-like genes in different tissues and in response to different biotic and abiotic stimuli is shown in Figure 2. Only three of the nine TCs (68378, 68095 and 60334) correspond to genes that are expressed in stems and are therefore candidates for involvement in stem lignification. There is no relation between sequence relatedness and expression pattern; for example, TC 68095 and 60334 are the most strongly expressed in stem, but do not cluster together based on sequence.

In contrast to the complex *Medicago PAL* gene family, tobacco contains two families of *PAL* genes (*PAL1* and *PAL2*), each family comprising two very closely related members. Protein gel blot analysis showed that PAL1 is localized in both soluble and microsomal fractions from tobacco stems and cell cultures, whereas PAL2 is only found in the soluble fraction (Rasmussen and Dixon, 1999). This suggests that PAL1 is the form of PAL involved in direct coupling to the membrane associated C4H (see below), and this has recently been confirmed by various *in vivo* immunocytochemical and immunofluorescence approaches coupled with laser confocal microscopy (L. Achnine, E. Blancaflor, S. Rasmussen and R.A. Dixon, unpublished results). It is possible that different forms of PAL with different sub-cellular localization may be associated differentially with S and G lignin and lignan biosynthesis, consistent with over-expression of a specific (transgene) PAL form affecting S/G ratio in transgenic tobacco (Sewalt et al., 1997). Such a channeled model for monolignol biosynthesis would allow for the fine control over production of G and S units that is required for the observed temporal and spatial regulation of lignin composition. Such a model does, however, still await definitive proof.

The data in Figures 2–6 show that many of the *PAL* and downstream monlignol pathway genes are modulated in response to abiotic and biotic stimuli, or expressed in tissue where lignification may not occur. This highlights the importance of the monolignol pathway for the synthesis of a range of end products with various functions including microbial and insect defense, establishment of interactions with pathogenic microorganisms, growth regulation, etc (Binns et al., 1987; Delay et al., 1994; Barber et al., 2000).

Cytochrome P450 enzymes (C4H, "C3H" and "F5H"). In contrast to PAL, the cytochrome P450 enzymes involved in monolignol biosynthesis (C4H, "C3H" and "F5H") each only appear to be encoded by one gene or a pair of genes in *M. truncatula*, of which one appears to be predominantly expressed in stems (Figure 3). Down-regulation of "C3H" and "F5H", and over-expression of "F5H", all have major impacts on lignin content and/or composition in other plants such as tobacco and Arabidopsis (Meyer et al., 1998; Franke et al., 2000; Franke et al., 2002). These effects are quantitatively greater than the effects of altering expression of any of the other enzymes in the pathway downstream of C4H, consistent with differences in the numbers of potential targets for down-regulation between each P450 and non-P450 enzyme in the pathway.

4-Coumarate: CoA ligase. *M. truncatula* appears to express at least 10 TCs that are annotated as encoding 4CL (Figure 4A, B). These may not all encode true 4CL enzymes acting on hydroxycinnamic acids, as 4CL-like enzymes are also known that utilize a similar reaction mechanism involving activation of an acidic function by formation of an acyl adenylate (Cukovic et al., 2001; Ehlting et al., 2001). None of the *M. truncatula* 4CL TCs has yet been functionally characterized, although distinct isoforms of 4CL have been enzymatically characterized in other species (Knobloch and Hahlbrock, 1975; Vincent and Nicholson, 1987; Lee and Douglas, 1996). Their biochemical properties indicate some degree of preference for the variously substituted hydroxycinnamic acids, but do not necessarily suggest specific cellular functions, e.g. in lignification.

Of the 10 *M. truncatula* 4CL ESTs, four are expressed in stem tissue, but none particularly highly when compared to the level of expression of TC69081 in yeast elicited cell cultures. Figure 4A shows a dendrogram of the *M. tuncatula* 4CL TCs summarizing sequence comparisons with other plant 4CLs in the GenBank database. Genes that have been targeted for lignin modification in transgenic plants are shown in bold. The contradictory effects on lignification of transgenically down-regulating 4CL in different, or even the same, species (Kajita et al., 1996; Lee et al., 1997; Hu et al., 1999), may well be a result of the complexity of the *4CL* gene family. None of the studies to date has used gene specific probes or arrays to determine the exact pattern of transcript down-regulation obtained for the different gene family members.

Hydroxycinnamoyl transferase (HCT). To date, only a single TC corresponding to HCT has emerged from

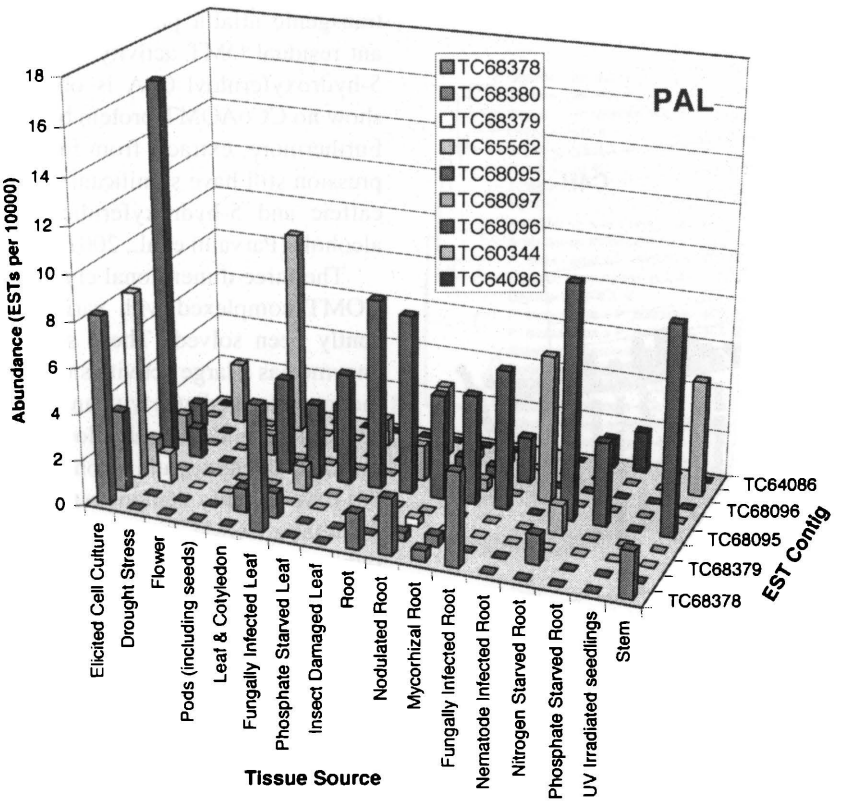

Figure 2. In silico expression analysis of *PAL* genes in *M. truncatula*. This species currently has close to 200,000 reported EST sequences, from nearly 40 different cDNA libraries, deposited in publicly available databases. EST sequences can be clustered into tentative consensus (TC) sequences, representing transcripts originating from one specific gene. In the case of PAL, nine such TCs currently exist. It is possible to query the database to determine the number of times an EST corresponding to a particular TC has been sequenced in a particular library or group of libraries. This number can then be expressed as a function of the total number of ESTs sequenced in that library(s) to give an approximate value for the expression level of the gene represented by that TC. See Dixon et al. (2002) for further details of this approach.

the *M. truncatula* EST sequencing projects. This appears to be expressed in all libraries examined to date, with the exception of UV-induced seedlings (Figure 5), and is therefore probably involved in multiple pathways involving hydroxycinnamate esters.

O-Methyltransferases. Figure 6 shows expression patterns of the *M. truncatula* caffeic acid *O*-methyltransferase genes. There are seven COMT-like TCs in *M. truncatula*, three of which are expressed in stems and therefore potentially involved in lignification in that organ (Figure 6). Interestingly, TC59577, the ortholog of the alfalfa COMT gene that has been shown, by transgenic down-regulation, to be essential for S lignin biosynthesis (Guo et al., 2000), is not the most highly expressed COMT gene in stem tissue. TC67955 is expressed at approximately 3.5 times the level of TC59577 in stem, and is also very strongly represented in leaf and flower cDNA libraries (Figure 6). TC67955 is most closely related at the DNA sequence level to the AEOMT (acids/esters OMT) from Loblolly pine, an enzyme reported to be active against caffeic and 5-hydroxyferulic acids and their corresponding coenzyme A esters (Li et al., 1997). However, TC67955, when expressed in either yeast or *E. coli*, appears to have no activity against caffeic acid or caffeoyl CoA, and preliminary results suggest no lignin phenotype on down-regulation or over-expression of the AEOMT-like gene in *Medicago* (P. Kota and R.A. Dixon, unpublished results). The existence of a novel *O*-methyltransferase gene(s) involved in monolignol biosynthesis in *Medicago* is predicted from the fact that strong down-regulation of both COMT and CCoAOMT in transgenic alfalfa is not additive and does not result in a strong reduction in lignin quantity (Inoue et al., 2000). Analysis of OMT substrate preferences in crude extracts from lignifying stems from wild-type and OMT down-regulated

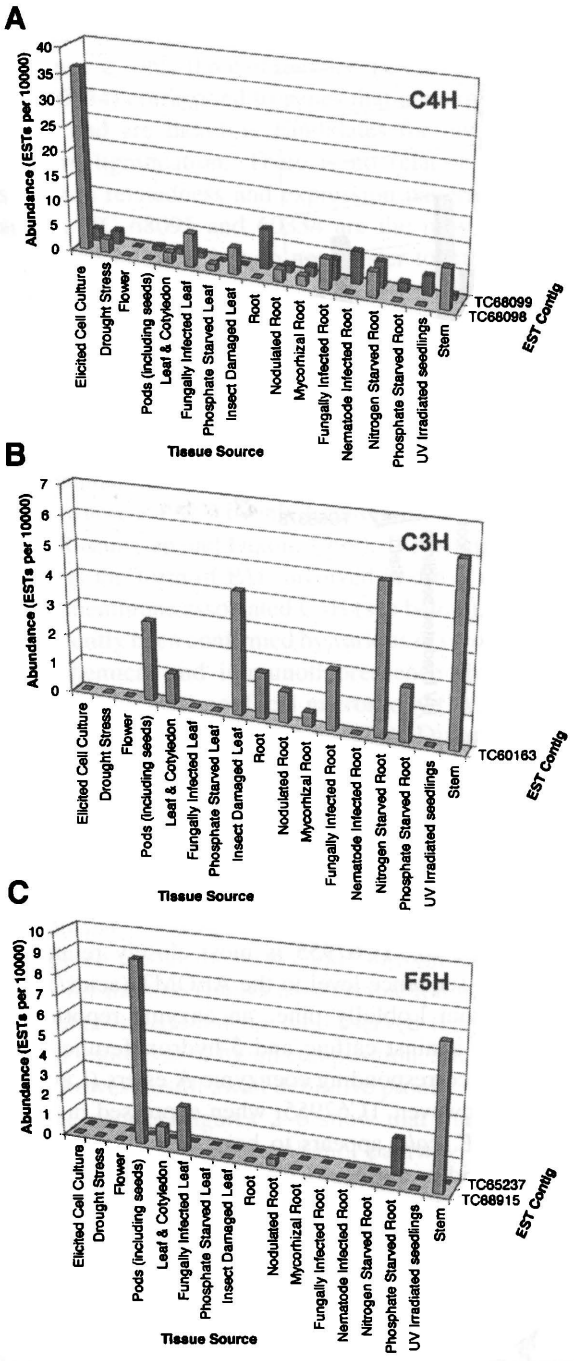

Figure 3. In silico expression analysis of *cytochrome P450* genes encoding cinnamate 4-hydroxylase, "coumarate 3-hydroxylase" and "ferulate 5-hydroxylase" in *M. truncatula.* See legend to Figure 1 for explanation.

transgenic alfalfa plants has indicated that significant residual OMT activity against caffeoyl CoA and 5-hydroxyferuloyl CoA is observed in extracts that show no CCoAOMT protein by western blot analysis. Furthermore, extracts from lines with no COMT expression still have significant residual activity against caffeic and 5-hydroxyferulic acids, aldehydes and alcohols (Parvathi et al., 2001).

The three dimensional crystal structures of alfalfa COMT complexed with various substrates have recently been solved. These studies indicate that the enzyme has a large activite site cavity that can accommodate intermediates with and without the 5-hydroxyl substituent, and that can allow for correct positioning of acids, aldehydes and alcohols (Zubieta et al., 2002). We have recently demonstrated that the best substrate for alfalfa COMT is not a monolignol precursor but rather the vanillin precursor protocatechuic aldehyde, related to caffeyl aldehyde by the shortening of the side chain by two carbons (P. Kota and R.A. Dixon, unpublished results). The physiological significance of this observation is not clear, but the finding highlights the danger in assigning *in vivo* function to an enzyme based solely on *in vitro* substrate preference.

M. truncatula contains four TCs annotated as encoding CCoAOMT. The tissue-specific expression patterns of these genes have been summarized elsewhere (Dixon et al., 2002). Two of these genes are expressed in stems. Similar complexity in CCoAOMT expression has been reported in tobacco, which contains a number of COMT and CCoAOMT isoforms with different substrate specificities and expression patterns (Maury et al., 1999).

Cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). Both CCR and CAD are members of gene families in *M. truncatula.* Of the six CCR TCs, two have so far been functionally expressed and shown to encode proteins active in the reduction of all potential CoA esters shown in Figure 1, although one form has a strong preference for feruloyl CoA (Q. Ma and R.A. Dixon, unpublished results). Of the seven apparent CAD TCs in *M. truncatula*, four are expressed in stems, and, as in the case of 4CL, the CAD form that appears to be most highly expressed in stem is also very strongly expressed in elicited cell cultures. To date, none of the *M. truncatula* CAD TCs has been functionally characterized. It is not therefore known whether *M. truncatula* contains a specific form of CAD that is preferentially involved in the reduc-

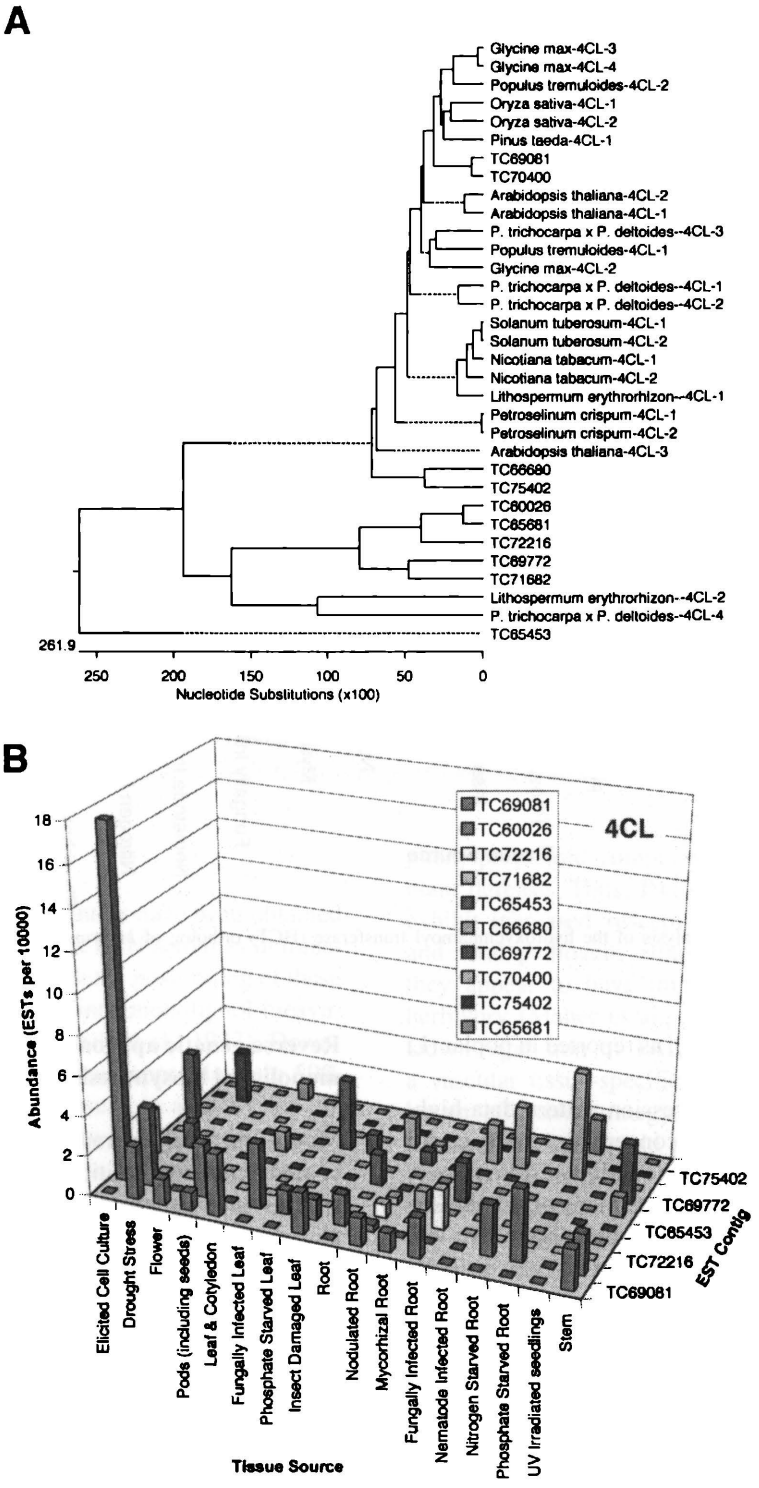

Figure 4. 4CL genes in *M. truncatula.* (A) Dendrogram showing relatedness among *M. truncatula 4CL* genes (based on TCs) and other known 4CL sequences. Sequences in bold represent genes that have been transgenically down-regulated to modify lignin content/composition. (B) *In silico* expression analysis; see legend to Figure 1 for explanation.

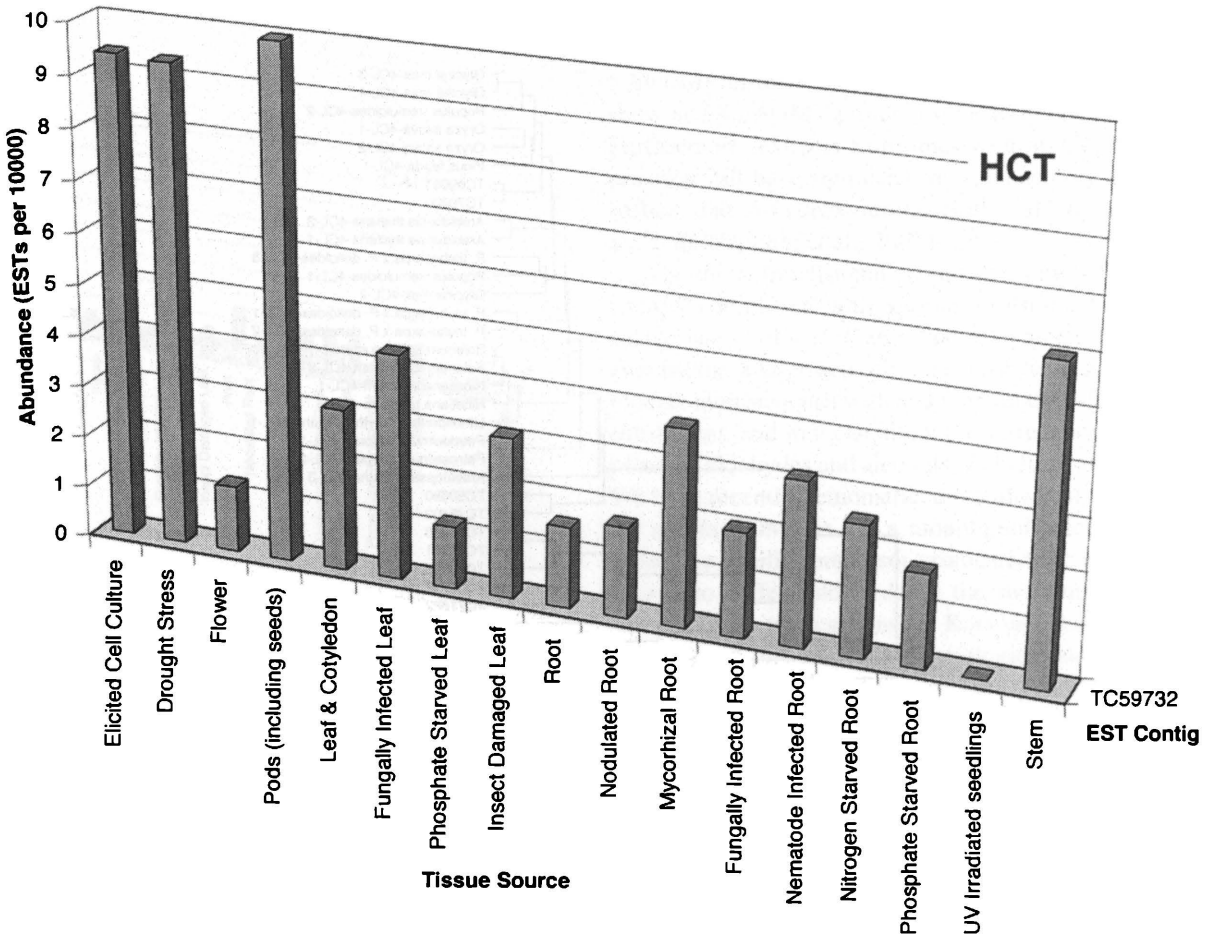

Figure 5. In silico expression analysis of the hydroxycinnamoyl transferase (HCT) ortholog of *M. truncatula*. See legend to Figure 1 for explanation.

tion of sinapaldehyde (SAD), as reported in poplar (Li et al., 2001).

The above *in silico* expression pattern data highlight two important points concerning strategies for genetic modification of lignin and lignans. First, only by knowing the sequence relatedness of all the expressed gene family members in the target plant will it be possible to predict the extent to which down-regulation of a particular enzymatic target will be complete. Second, many of the enzymes of lignin/lignan biosynthesis are expressed in a range of tissues, and in some of these their function may not necessarily be in lignification or lignan formation. Therefore, it may be advantageous to avoid constitutive down-regulation.

Reverse genetic approaches to understanding monolignol biosynthesis

Most of the work on transgenic manipulation of lignin content and composition has been done in model species such as tobacco, Arabidopsis and tree species such as poplar and Eucalyptus. Although the biosynthetic pathways to the monolignols, and the mechanisms of monolignol polymerization, are most likely conserved among plant species, there are clearly species-specific differences in the fine regulation of these processes. These basic similarities and specific differences are apparent on reviewing the now quite large body of literature describing transgenic modifications to the monolignol pathway but, unfortunately, there is as yet no analysis of the impact of sequential down-regulation of each step in the monolignol pathway in a single species.

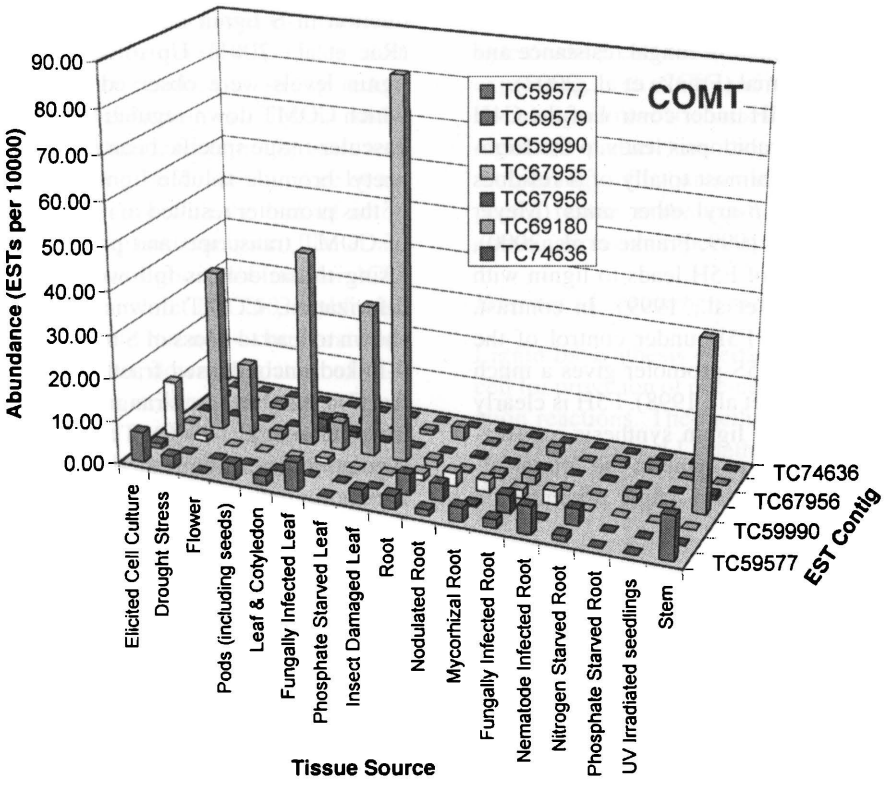

Figure 6. In silico expression analysis of *COMT*-like genes in *M. truncatula.* See legend to Figure 1 for explanation.

PAL and C4H

Large reductions in lignin content have been obtained by down-regulation of PAL or C4H activities in transgenic tobacco, consistent with the positioning of these enzymes at the entry point into monolignol biosynthesis (Bate et al., 1994; Sewalt et al., 1997). Down-regulation of PAL leading to an approximately 50% reduction in Klason lignin results in a near doubling of S/G ratio. In contrast, down- regulation of C4H leading to a similar decrease in lignin content results in greatly reduced S/G ratio, tending towards complete elimination of S units (Sewalt et al., 1997). This is quantitatively similar to the loss of S lignin observed following down-regulation of COMT (Atanassova et al., 1995; Van Doorsselaere et al., 1995) or F5H (Chapple et al., 1992) but none of the other enzymes of monolignol biosynthesis. The reason(s) for the opposite effects of down-regulation of PAL and C4H on lignin composition have yet to be determined.

Constitutive down-regulation of PAL or C4H, although an effective approach for reducing lignin content, is unlikely to find practical application. This is because of the pleiotropic effects on levels of other phenylpropanoid compounds that are important for plant defense. Thus, PAL-suppressed tobacco plants exhibit increased susceptibility to fungal, bacterial, and viral pathogens although, perhaps surprisingly, they appear to have improved resistance to insect herbivory (Maher et al., 1994; Felton et al., 1999). Down-regulation of PAL or C4H under control of a vascular tissue-specific promoter might, however, be an effective strategy, assuming that other non-lignin phenylpropanoid compounds produced in the vascular elements are not required for plant health or development.

C3H and F5H

The *ref8* mutant of Arabidopsis lacks expression of CYP98A3, the enzyme involved in the 3-hydroxylation of hydroxycinnamate esters. Although initially identified based on the lack of sinapate esters in the leaves, this mutant has an unusual lignin built primarily from 4-coumaryl alcohol, consistent with the block in 3-hydroxylation. However, like PAL down-regulated plants (Felton et al., 1999), the mutation causes pleiotropic effects due to reduction

in important non-lignin phenylpropanoid compounds, and is consequently impaired in fungal resistance and is developmentally abnormal (Franke et al., 2002).

Over-expression of F5H under control of the C4H promoter in transgenic Arabidopsis leads to the formation of lignin composed almost totally of S residues linked predominantly in β-aryl ether units (Meyer et al., 1998; Marita et al., 1999; Franke et al., 2000), whereas down-regulation of F5H leads to lignin with very few S units (Marita et al., 1999). In contrast, constitutive expression of F5H under control of the cauliflower mosaic virus 35S promoter gives a much weaker phenotype (Meyer et al., 1998). F5H is clearly a major control point in S lignin synthesis, consistent with the position of the enzyme in the "classical" pathway and its revised position acting primarily on 5-hydroxyconiferaldehyde.

OMTs

There have been several reports on the effects of down-regulation of COMT activity on lignin content and composition in transgenic plants, and the *brown mid-rib3* mutation in maize results from the insertion of a retrotransposon in the *COMT* gene (Vignols et al., 1995) with the phenotype being reproduced by targeted down-regulation of COMT (Piquemal et al., 2002). Although the first study on COMT down-regulation in transgenic tobacco plants reported reductions in lignin content with no significant changes in lignin composition (Ni et al., 1994), it has since been consistently observed that strong down-regulation of COMT in species as diverse as tobacco and poplar results in a drastically reduced S/G ratio due primarily to inhibition of S lignin biosynthesis (Atanassova et al., 1995; Van Doorsselaere et al., 1995). Furthermore 5-hydroxyguaiacyl residues accumulate in the lignin of COMT down-regulated plants (Atanassova et al., 1995; Van Doorsselaere et al., 1995). Although reduction in S units is the major feature of lignin in COMT down-regulated plants, reduction of COMT activity in the xylem of quaking aspen (poplar) also results in a red-brown coloration resulting from high amounts of coniferaldehyde (Tsai et al., 1998). Furthermore, strong down-regulation of COMT in transgenic poplar resulted in a significant (17%) decrease in overall lignin level in 6-month old trees (Jouanin et al., 2000).

35S promoter-driven antisense reduction of COMT to less than 5% of wild-type values in the tropical pasture legume *Stylosanthes humilis* resulted in no apparent reduction in lignin levels but in a strong reduction in S lignin based on histochemical analysis (Rae et al., 2001). Up to 30% decreases in Klason lignin levels were observed in transgenic alfalfa in which COMT down-regulation was targeted using the vascular-tissue specific bean *PAL2* promoter, although acetyl bromide soluble lignin was not reduced. Use of this promoter resulted in near total down-regulation of COMT transcripts and protein (Guo et al., 2000). Using thioacidolysis followed by Raney nickel desulfurization, COMT down-regulation in alfalfa was shown to lead to a loss of S residues in both the $\beta-O$-4-linked uncondensed fraction and in the condensed fraction resolved as a range of differently linked dimers (Guo et al., 2000). The effect of COMT down-regulation on S lignin therefore likely reflects a true metabolic reduction in S units, rather than a change in lignin composition resulting in appearance of more S units in the non-condensed fraction of the polymer. Loss of S-units was accompanied by appearance of thioacidolysis-determined 5-hydroxyguaiacyl residues in the lignin, and the presence of these residues, and their linkage to yield novel lignin benzodioxane units, has been confirmed by the use of 2-dimensional NMR techniques (Marita et al., 2002).

In tobacco, down-regulation of CCoAOMT leads to a corresponding decrease in Klason lignin levels, associated with a reduction in both S and G lignin monomers (Zhong et al., 1998). This finding, when considered in relation to the strong reduction of S lignin in COMT down-regulated plants, has been interpreted as indicating that CCoAOMT is involved in 3-*O*-methylation during the formation of both G and S monolignols, whereas COMT functions primarily in 5-*O*-methylation during S lignin biosynthesis. However, similar comparisons of effects of COMT and CCoAOMT down-regulation in alfalfa suggest that this is not necessarily true in all plants. Near elimination of CCoAOMT activity reduced G lignin by up to 50% in some alfalfa lines, but with no effect on S lignin (Guo et al., 2000).

4CL

The results of down-regulation of 4CL on lignin content and composition are somewhat contradictory. In tobacco, a reduction of 4CL activity resulted in a brownish coloration of the xylem cell walls, which correlated with reductions in overall lignin levels and in cinnamyaldehyde and S residues in lignin. However, values for S/G ratio varied widely, from 0.45 to 2.0, in plants with reduced 4CL activity due to

large but variable reductions in G lignin (Kajita et al., 1996). In Arabidopsis, reduction of 4CL to less than 10% of wild-type activity resulted in strong reduction in G lignin with no reduction in S lignin, and it was therefore suggested that a route might exist to S lignin that is independent of 4CL activity (Lee et al., 1997). This idea is consistent with the lack of activity of Arabidopsis 4CL against sinapate (Lee et al., 1997). However, sinapate is a good substrate for 4CLs from other species (Yamauchi et al., 2003), although a 4CL that accepts sinapate but not 4-coumarate has not been reported. In poplar, down-regulation of 4CL was reported to result in a striking decrease in lignin content with no apparent change in lignin composition (Hu et al., 1999). Interpretation of the results of 4CL down-regulation is difficult in the absence of complete knowledge of the full range of 4CL isoforms in the target organism, and their relative substrate specificities.

CCR and CAD

Down-regulation of CCR in tobacco leads to an orange/brown xylem coloration, reductions in lignin content, and increased S/G ratio due primarily to a decrease in extractable G units (Piquemal et al., 1998). However, there was some variation between lines, and one line also showed a significant reduction in S units. These data would, therefore, be consistent with the involvement of CCR in the synthesis of both G and S lignin. Down-regulation of CAD in tobacco leads to lignin with an increased cinnamaldehyde content (Halpin et al., 1994; Hibino et al., 1995; Stewart et al., 1997), and increased aldehyde levels are also observed in the lignin of a loblolly pine mutant that is severely depleted in CAD (Ralph et al., 1997). It should be noted that the effects of altered CCR or CAD on S/G ratio reported to date are far less striking than the effects of altered F5H or COMT. One explanation would be that F5H and COMT are specific for S lignin biosynthesis, whereas CAD and CCR are common to both S and G lignin biosynthesis.

Antisense down-regulation of CAD in transgenic alfalfa to approximately 30% of wild-type level leads to a red coloration of the stem and a reduction in S/G ratio primarily due to a decrease in S units (Baucher et al., 1999). Recent studies have examined the effects of simultaneous down-regulation of both CCR and CAD in transgenic tobacco (Chabannes et al., 2001; Chabannes et al., 2001). Whereas strong down-regulation of CCR in tobacco results in a phenotype characterized by dramatic structural abnormalities to xylem vessel cell walls (Piquemal et al., 1998), plants expressing both CCR and CAD transgenes had reduced lignin levels characteristic of the CCR down-regulated parent line, but normal xylem cell wall morphology (Chabannes et al., 2001). These unexpected results make examination of other transgene combinations an important priority.

Enzymes of one carbon metabolism

Lignin biosynthesis exerts a high requirement on the cell for provision of one-carbon groups for the methylation reactions. The methyl group donor in lignification is S-adenosyl L-methionine (SAM), and production of this molecule occurs via a cyclical process (the active methyl cycle) in which the S-adenosyl homocysteine produced in the COMT and CCoAOMT reactions is first hydrolyzed to homocysteine which is then methylated to methionine prior to re-activation by SAM synthetase. It is interesting to note that SAM synthetase is among the most highly expressed genes in *M. truncatula* based on EST counts in the 40 different libraries sequenced to date. Genetic modification of SAM synthetase expression can have a profound effect on lignification. Thus, a single point mutation in the ATP binding domain of the *Arabidopsis SAMS3* gene results in an approximately 35% decrease in intercellular SAM pools and a corresponding 22% decrease in lignin content (Shen et al., 2002). However, because SAM is an important methyl group donor in other cellular processes, and reductions in SAM levels result in undesirable phenotypes (Masuta et al., 1995), modification of the SAM cycle may be of limited value for lignin modification as an agronomic trait.

Summary

It is interesting that, after more than 40 years of study, our understanding of monolignol biosynthesis is still evolving. Molecular genetics and genomics approaches, coupled with advanced analytical chemistry techniques, will provide tools for obtaining an unequivocal understanding of the pathways that lead to hydroxyphenyl, guaiacyl and syringyl monolignols and their polymerization products. Important questions that still remain to be answered include:

- Does monolignol biosynthesis proceed via a grid or through linear pathways?
- How is independent regulation of G and S synthesis achieved?

- Is there enzymatic redundancy at key steps in monolignol biosynthesis?
- Do enzyme isoforms encoded by different gene family members have independent functions?
- What is the extent and physical nature of metabolic channeling within the monolignol pathway?

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