Assembly of a lignin modification toolbox

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Abstract

This project focused on establishing an enzymatic toolbox for the production of lignin modification molecules (LMMs). We took multiple approaches to the identification and characterization of LMM-synthesizing enzymes and identified three classes of catalysts that were the focus of our work.

Our first objective was to gain a greater understanding of structure-function relationships in cytochrome P450-dependent monooxygenases (P450s), enzymes required for the introduction of important functional groups on lignin precursors. The analysis of a number of mutants and transgenic plants has demonstrated the role that P450s play in determining lignin monomer composition.[1-4] First, we generated a large family of chimeric P450 proteins by recombining cinnamate 4-hydroxylase (C4H) genes from distantly related plant species. Many of these proteins retained their enzymatic activities, strongly supporting the idea that chimeric P450s can be generated using a library of less-closely-related genes to generate novel catalysts. Following up on the success of this approach, we are completing additional chimeric libraries between P450s which catalyze different steps in lignin synthesis. We next focused on C4H and ferulate 5-hydroxylase (F5H) from Arabidopsis, but the chimeric libraries generated from these proteins generated largely inactive proteins, suggesting that they are too distantly related to be used as parents for successful chimeragenesis. A more likely combination of new activities is likely to be derived from a combination of F5H and a paralog of F5H we identified that is required for the synthesis of a group of novel alpha-pyrone secondary metabolites accumulated in Arabidopsis stems. This new enzyme has recently evolved from an enzyme in lignin biosynthesis and may provide us with another tool with which to modify lignin biosynthesis directly and will give us greater insights into the evolution of substrate specificity in P450s.

Another group of proteins known as BAHD acyltransferases use Coenzyme A (CoA) thioesters as activated donors in acyltransferase reactions that generate dimeric phenylpropanoid conjugates. These products can be regarded as potential LMMs because their phenolic moieties are amenable to lignin incorporation, while their internal ester or amide bonds would introduce readily cleavable linkages into the lignin polymer. As a focus of this project we successfully isolated the full length sequence of a BAHD acyltransferase from the Asian fiber crop kenaf (*Hibiscus cannabinus*), which is known to accumulate the candidate LMM coniferyl ferulate.

Like Coenzyme A thioesters, 1-*O*-glucose esters such as 1-*O*-sinapoyl-β-D-glucose have a high free energy of hydrolysis such that they can serve as activated acyl donors. We have focused on a family of serine carboxypeptidase-like (SCPL) proteins that are acyltransferases that use sinapoylglucose for the synthesis of sinapic acid esters including the LMM candidate 1,2-disinapoylglucose. Using GCEP funding, we have shown that two SCPL proteins from Arabidopsis are required for sinapoylation and benzoylation of glucosinolates in seeds. In addition, we have over-expressed an SCPL protein that synthesizes a candidate LMM in Arabidopsis and have found that this strategy significantly enhances the accumulation of this candidate LMM in leaves and also dramatically alters the subcellular localization of the most abundant sinapic acid esters accumulated in the these plants.

Finally, during the latter portion of the funding period of this project we initiated a new project to identify potential LMMs through the analysis of monolignol conjugates in natural accessions of Arabidopsis. These new metabolites are themselves potential LMMs. The acquisition of leaf metabolite data on 440 accessions was completed and the analysis of stem metabolites will be continued with other funding. Using genome-wide association mapping, we expect to be able to make rapid progress toward the identification of the enzymes and genes involved in their synthesis, although the culmination of this project will extend beyond the GCEP funding period.

Introduction

Although the phenylpropanoid pathway produces many compounds of value to humans, a major goal of research on the pathway has been to improve our understanding of lignin biosynthesis.[5] Biofuel production has provided a new motivation for this interest because the quantity and quality of lignin in biomass crops interferes with the access of hydrolytic enzymes to the polysaccharide components of the plant cell wall, thereby inhibiting their conversion to fermentable monosaccharides. For this reason, the ability to manipulate lignification in biofuels crops would lead to substantial economic and environmental gains. The objective of this project was to identify a set of enzymes with which we could fundamentally alter the chemistry of the lignin polymer in such a way that it would radically alter the ease with which lignin can be removed from biofuel crops. The long term objective of this effort was to substantially increase yields of cell wall-derived sugars and the efficiency of their subsequent fermentation without compromising the viability of the plants themselves.

As a result of their involvement in many secondary metabolic pathways, P450s as well BAHD and SCPL acyltransferases can be found in a wide range of plants, and exhibit broad catalytic diversity. We expect that when these native or engineered genes are over-expressed in biofuel crops, the phenolic nature of the LMMs they produce will lead to their incorporation into lignin but will not compromise the function of this important cell wall component. Either through altered cross-linking or through the incorporation of ester or amide linkages, the LMMs will generate “weak links” within the lignin polymer, greatly enhancing the ease with which it can be deconstructed post-harvest.

Background

The lignin heteropolymer is produced via the oxidative coupling of *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol subunits (collectively termed monolignols). The polymerization of these subunits leads to the formation of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin, respectively. H subunits are usually minor components, and the degree to which G and S units are incorporated into the polymer (commonly denoted as the S:G ratio) varies widely among species, tissue types, and even within an individual cell wall. Although H, G, and S units are widely regarded as the only monomers found in lignin, more sophisticated methods of lignin analysis applied to a broader range of plant species, mutants, and transgenic lines have revealed that other subunits, including aldehydes, side-chain-reduced monolignols, and phenylpropanoid esters and amides appear to be *bona fide* lignin components.[6-8]

This plasticity of lignin monomer composition is further demonstrated by the *fah1* mutant of Arabidopsis, the lignin of which lacks syringyl monomers [1], maize and pine mutants that deposit lignins containing 5-hydroxyguaiacyl and dihydroconiferyl alcohol monomers, respectively [9-10], engineered Arabidopsis, tobacco, and poplar lines that produce lignins derived almost solely from syringyl units [2-3], and an Arabidopsis mutant that deposits lignin containing only *p*-hydroxyphenyl units [3,11], among others. It is noteworthy that in some cases, these modified lignins are much more readily extracted than the lignins normally made by plants [12], a result that bodes well for the utility of lignin modification strategies in the improvement of biofuel crops.

Based upon our emerging understanding of the plasticity of lignin monomer composition, it seems clear that it will be possible to engineer plants to generate novel lignins that still support normal plant growth, development, and levels of biomass productivity. This might be accomplished by engineering plants to synthesize monolignols with novel substitution patterns that would lead to lignins with altered chemistries and enhanced degradability. Alternatively, there is ample precedent in the literature to suggest that plants could be engineered to incorporate dimeric phenylpropanoid ester- or amide-linked conjugates into their lignin, making it easier to cleave either chemically or enzymatically. Both of these strategies would result in a polymer that can be more effectively and efficiently removed from biomass feedstocks.

Results

BAHD acyltransferase proteins

BAHD family acyltransferases can be phylogenetically grouped into several distinct clades, one of which contains a number of enzymes which utilize hydroxycinnamic and benzoic acid derivatives as substrates. Members of this clade are particularly promising as potential LMM-synthesizing enzymes, since hydroxycinnamic and benzoic acid derivatives are frequently incorporated into the lignin polymer. We have successfully employed a degenerate PCR-based approach using conserved amino acid sequences from BAHD family members to obtain the full length clone for a novel acyltransferase-encoding gene from the Asian fiber crop kenaf (*Hibiscus cannabinus*), which is known to accumulate the candidate LMM coniferyl ferulate. Phylogenetic analysis strongly suggests that the closest relatives of the acyltransferase is involved in the synthesis of

benzyl benzoate or phenethyl benzoate, both of which are strikingly similar in structure to conferyl ferulate. Further characterization of the enzyme is currently in progress.

SCPL enzymes

To understand structure-function relationships within the SCPL proteins, we have initiated reverse genetic analyses of Clade I SCPL genes. [13] We focused on six genes showing preferential expression in seeds for two reasons. First, the genes encode proteins belonging to uncharacterized sub-groups of Clade I. Second, and most importantly, seeds contain a diverse group of sinapoylated metabolites and the enzymes that synthesize them may prove useful in the development of LMMs. Knock-out (KO)

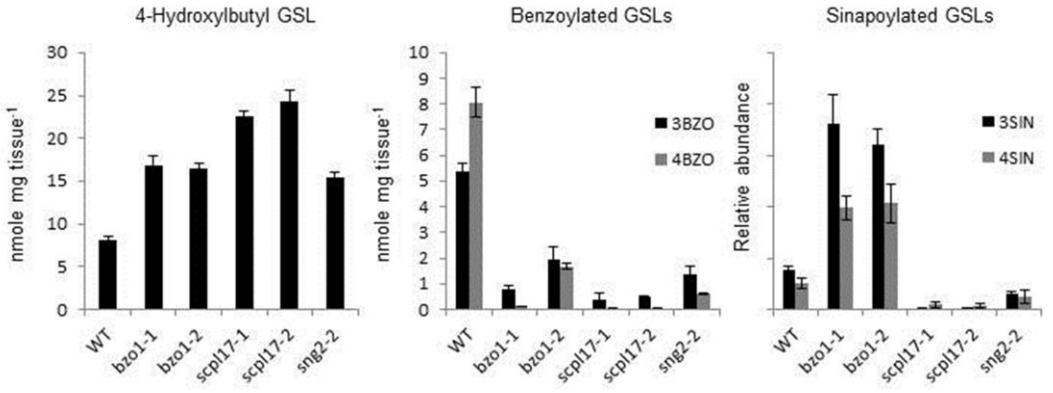

Figure 1: Glucosinolate analyses in *scpl17*, *scpl17*, and *sng2* mutant seeds. Both benzoylated and sinapoylated glucosinolates were dramatically reduced in *scpl17* and *sng2* mutants while 4-hydroxybutyl glucosinolate increased. Error bars indicate standard deviations of four replicates.

lines were identified for five SCPL genes, and RNAi lines were generated for a further two. Among single KO or RNAi seeds, only *scpl17* KO and *scpl19* KO seeds showed changes in putatively sinapoylated compounds in total seed metabolite analyses. Based on gene co-expression data available in publically accessible databases, we speculated that in seeds, these SCPL proteins might be involved in the benzoylation or sinapoylation of hydroxylated glucosinolates. Glucosinolates were analyzed in the KO or RNAi lines and both benzoylated and sinapoylated glucosinolates decreased to almost undetectable levels in *sng2* and *scpl17* mutants (Fig. 1). Further quantitative analyses showed that the levels of precursor hydroxylated GSLs increased while the levels of the other major GSLs were not changed. This finding suggests that both SCPL17 and SCPL19 may be involved in both sinapoylation and benzoylation of GSLs, or that there is an interaction between these two acylation pathways at the metabolic or regulatory level. This work was submitted to The Plant Journal for publication and received favorable reviews. A revised manuscript addressing reviewers' concerns is about to be resubmitted.

We have also initiated an attempt to directly test the feasibility of generating hydrolysable lignin units through the overexpression of sinapoylglucose:sinapoylglucose sinapoyltransferase (SST), an enzyme required for the production of a candidate LMM, 1,2-disinapoylglucose (DSG). In a wild-type background, the was virtually no increase in the amount of DSG accumulated, probably because of the competing activity of sinapoylglucose:malate sinapoyltransferase (SMT), which takes common acyl-donor sinapoylglucose to synthesize sinapoylmalate. In contrast, when we overexpressed *SST* in a SMT-deficient mutant background we observed significant increase of DSG and another disinapoylated monosaccharide in leaves.

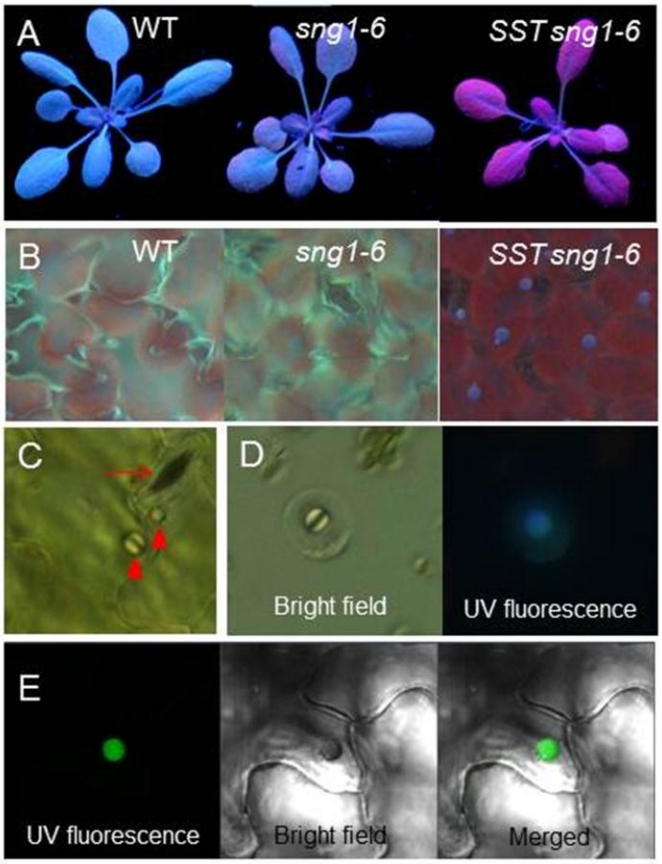

Figure 2: Characterization of SST overexpressing SMT-deficient plants. (A) UV fluorescence of 3-week old plants. (B) UV fluorescence of the 7th leaves of 3-week old plants under microscope. (C) Adaxial epidermis of a *SST sng1-6* leaf. The arrow indicates a stomata. Two arrowheads indicate the putative fluorescent particles under bright field. (D) Bright field and UV fluorescence images of a *SST sng1-6* protoplast. (E) Confocal microscope images of adaxial epidermis of a *SST sng1-6* leaf. UV fluorescence was artificially colored with green.

Surprisingly, we found that in the SST overexpressing plants, there was a modest decrease in soluble phenylpropanoid content compared to wild type and a change in the sub-cellular distribution of sinapoylated compounds (Fig. 2). Microscopic observation of leaves showed that while the UV fluorescence of sinapoylmalate was localized to the vacuoles in wild type, the blue fluorescence of DSG was localized to discrete sub-

vacuolar compartments in SST-overexpressing plants. Neutral red staining showed that the particles stained much more strongly than the surrounding vacuolar lumen indicating that these subvacuolar bodies are more acidic environments than the organelles in which they are found. These observations, while unexpected, further demonstrate the importance of studying the subcellular localization of enzymes and products of the phenylpropanoid pathway in the context of lignin modification. This work is currently being written up for publication.

Cytochrome P450-dependent monooxygenases

We have developed high-throughput *in vivo* assays for the P450 enzymes that are involved in phenylpropanoid metabolism derived from *Arabidopsis thaliana* including C4H, F5H, and *p*-coumaroyl shikimate 3' hydroxylase (C3'H) and C4H and F5H from *Selaginella moellendorffii*. Each of these assays are conducted in heterologous WAT11 yeast strains that also express the corresponding P450 reductase from Arabidopsis. We have validated this method for C4H activity to ensure its reproducibility, robustness, and sensitivity in the large-scale assay of activity for more than a thousand chimeric protein variants that we expect to generate. After combining a number of small modifications, the sensitivity of this assay has been increased more than 10-fold, yielding an assay that has a dynamic range of more than 100.

Hybrids from site-directed chimeras between the C4H enzymes from *Arabidopsis thaliana* and *Selaginella moellendorffii* have been characterized for activity in vivo. Many of them retain significant activity. We have also taken the in vivo activity data and used it to generate a model of the origins of C4H activity. We find that, using only five parameters, the species identity of four of the fragments and a score derived from our graphical models of amino acid interactions, we can develop a linear model that explains a highly significant fraction of the total variation in activity among the chimeras. The ability to conduct such analyses should aid future engineering of novel P450's.

We have also completed the first library between two different P450 enzymes, the C4H and F5H of *Arabidopsis,* and are preparing to transfer these chimeras into yeast and assay their activity as both C4H and F5H enzymes and their ability to hydroxylate novel substrates. Although these chimeras are largely inactive, we expect that chimeras generated using P450s with more similar substrates or chimeras between enzymes with more recent common ancestors, will generate active enzymes.

Identification of a novel phenylpropanoid P450 in Arabidopsis

F5H (CYP84A1) catalyzes the 5-hydroxylation of coniferaldehyde and coniferyl alcohol to yield 5-hydroxy-coniferaldehyde and 5-hydroxy-conferyl alcohol, respectively. [2,5] In Arabidopsis, there is a paralog of CYP84A1 that has been designated CYP84A4. When we heterologously expressed CYP84A4 we found that it did not show detectable activity towards either coniferyl alcohol or coniferyl aldehyde, both of which are substrates of CYP84A1. Based upon these data, we concluded that the catalytic activity of CYP84A4 is distinct from that of CYP84A1 and that CYP84A4 has lost its ancestral activity as an F5H. CYP84A4 appears to be recently evolved in Arabidopsis because few angiosperms have CYP84 family members other than those that encode F5H, and specifically, a CYP84A1 paralog like CYP84A4 is not present in members of the closely

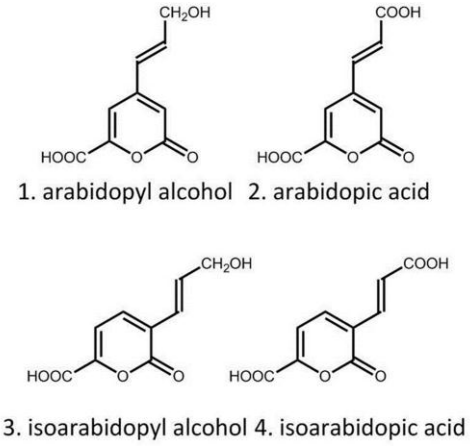

Figure 3. APs identified in Arabidopsis.

related Brassica genus. To elucidate the function of CYP84A4, we first identified a homozygous CYP84A4 T-DNA insertional mutant (Salk_064406), which we later named *apd1-1* for in *arabidopyrone deficient1*. Plants homozygous for *apd1* accumulate normal levels of sinapate esters which, together with the strong phenotype of the CYP84A1-deficient *fah1* mutant, indicate that CYP84A1 and CYP84A4 are not redundant genes. Reverse genetics and metabolic profiling have revealed that CYP84A4 is required for the synthesis of four previously unknown metabolites that we have named arabidopyrones (APs). To determine the identity of the unknown compounds we purified them by HPLC, analyzed them by LC-MS, and elucidated their structures by NMR. To our surprise, the metabolites had not been previously described in Arabidopsis or any other plants to date. The four metabolites are typified by a 6-carboxy-2-pyrone core moiety, decorated with either a 3-hydroxyprop-1-en-1-yl or a 2-carboxyvinyl side chain at the 3 or 4 position of the pyrone ring, reminiscent of the side chain of the general phenylpropanoid alcohols or acids (Fig. 3). We have named these newly discovered pyrone compounds arabidopyl alcohol, *iso*-arabidopyl alcohol, arabidopic acid and *iso*-arabidopic acid. The structure of arabidopyl alcohol was confirmed by synthesis.

When we assayed heterologously-expressed CYP84A4 against a range of possible phenylpropanoid pathway intermediates, only *p*-coumaraldehyde was found to be a substrate for CYP84A4, yielding caffeylaldehyde as a product. In view of these results it is interesting to note that CYP84A1, which is presumably the ancestral protein from which CYP84A4 is derived, exhibits slight activity as a *p*-coumaraldehyde and *p*-coumaryl alcohol 3-hydroxylase with Km values several hundred fold higher than those for its optimal substrates, coniferaldehyde and coniferyl alcohol. This novel catalyst provides an important additional input for future chimeragenesis work, and may shed light on how substrate specificities can evolve among the P450 superfamily.

Gaining access to novel phenylpropanoid metabolism in Arabidopsis

We have developed methods for the LC-MS analysis of a group of novel phenylpropanoids in Arabidopsis (Fig. 4) which hyper-accumulate in a mutant that is now the focus of another proposal pending with GCEP. [14] A striking commonality observed in these structures is that they include a hydroxycinnamyl alcohol moiety that appears to have been involved in a radical-coupling reaction reminiscent of those involved in lignin polymerization. We are now using our LC-MS based profiling strategy

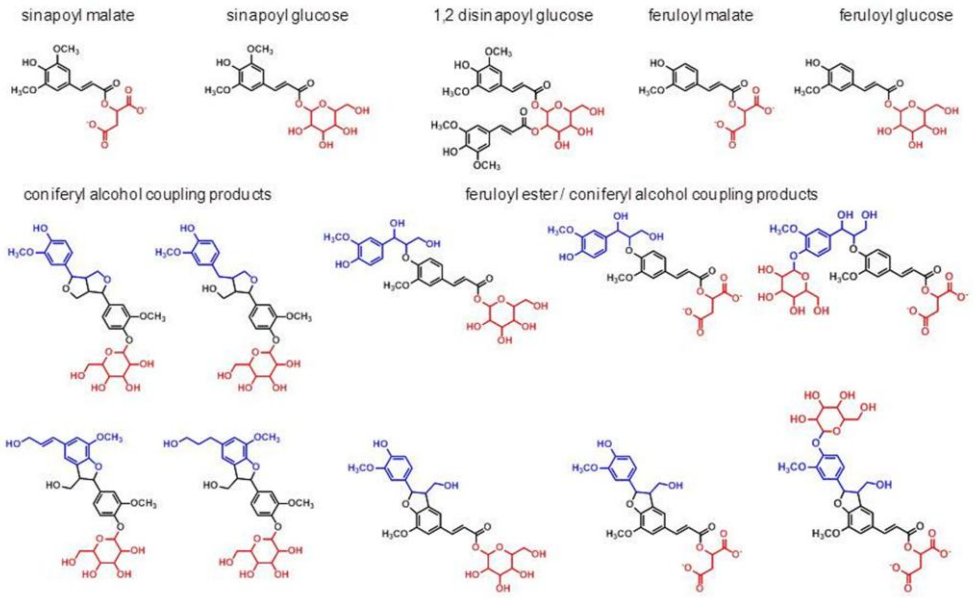

Figure 4. Novel monolignol coupling products identified in Arabidopsis. Known metabolites are shown in the top row, with hydroxycinnamic acids shown in black and alcohol acceptors (malate or glucose) shown in red. In the bottom two rows, novel cross-linking products are illustrated with their monolignols moieties shown in blue.

to identify Arabidopsis natural accessions that are altered in their accumulation of lignans and monolignol conjugates. Using genome-wide association mapping, we will be able to identify genes required for their synthesis in only 1-2 months after the profiling is complete. The enzymes encoded by these genes will then be characterized in detail, and hold distinct promise as catalysts to generate useful LMMs.

Conclusions

In this report, we have outlined our efforts towards isolating genes of potential value in the production of LMMs, lignin building blocks that we anticipate will improve the degradability of lignin while leaving intact its critical function in the cell wall. Specifically, we have made major steps towards the identification of novel P450s that could be used to modify lignin biosynthesis. We have also identified genes encoding novel acyltransferases that have the capacity to redirect phenylpropanoid metabolism as well as a suite of endogenous putative LMMs whose synthesis will soon be elucidated. It is our hope that when these discoveries, or those derived from them, are deployed in

biofuel crops, they will vastly increase the efficiency of cellulosic biofuel production and dramatically curb our excessive emission of greenhouse gasses.

As promised, we have added a significant number of genes and enzymes to our "lignin modification toolbox". Further, as anticipated in this high-risk, high-reward research, the emergent properties associated with biological systems dictate that results directly related to lignin modification and improved bioenergy production are likely to require long term investment in order to pay dividends. For example, although we were able to significantly increase the accumulation of DSG in Arabidopsis, this had the unexpected effect of altering the subcellular localization of this putative LMM. More work will be required to understand and gain control of the machinery that sequesters phenylpropanoids in various compartments before its accumulation can be directed to the plant cell wall where it can have its desired effect through incorporation into the lignin polymer. Similarly, although many of our C4H chimeras are active, active chimeras made with more distantly related P450s remain elusive, suggesting that it we will need to pursue further work with P450s of more closely related substrate specificity and/or phylogenetic relation in order to generate novel active enzymes. Finally, we have found that another opportunity for lignin modification may lie in plants' own biochemical diversity. By identifying the enzymes required to make the small monolignol conjugates we have identified in Arabidopsis, we may be able to upregulate and redirect their synthesis to the cell wall as an additional mechanism for lignin modification.

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