

Assembly of a lignin modification toolbox

Investigators

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Abstract

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

First, the analysis of a number of mutants and transgenic plants has demonstrated the role that cytochrome P450-dependent monooxygenases (P450s) play in determining lignin monomer composition.[1-4] We predict that the isolation of additional P450s that lead to unique hydroxylation patterns in monolignols will generate lignins with novel chemical properties that may improve lignin degradability. To date, we have established robust expression and analysis methods for recombinant plant P450s and are currently evaluating the substrate specificities of 25 heterologously expressed P450s. In parallel we are evaluating the metabolic profiles of Arabidopsis lines in which the genes encoding these same P450s have been inactivated. Using this approach, we have identified a P450 that is involved in the synthesis of a major metabolite accumulated in Arabidopsis roots, the identification of which is currently underway. Furthermore, we have established high-quality alignments of a large family of P450 proteins from a range of species that we are now using to direct chimeric protein construction to explore structure/function relationships in these important proteins. The analysis of these chimeric proteins will be supported by the robotic transformation and assay methods that we have developed over the past year.

Second, the 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. So far, we have established high-throughput platforms for expression of BAHD acyltransferases and for characterization of their enzymatic activities. We have completed an extensive bioinformatic analysis of BAHD acyltransferases from a number of model species that will guide us in the selection of enzymes to be examined in detail, and we are currently exploring PCR-based methods to retrieve as-yet-uncharacterized BAHD acyltransferase genes from species that are known to make compounds of interest as LMMs.

Finally, 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 identified a family of serine carboxypeptidase-like (SCPL) proteins that are in fact acyltransferases that use sinapoylglucose for the synthesis of sinapic acid esters including the LMM candidate 1,2-disinapoylglucose. We predict that expression of SCPL acyltransferases in lignifying tissues will generate LMMs with internal ester linkages that will be incorporated into the lignin polymer and vastly improve the ease with which lignin can be degraded. Our progress on this objective includes the demonstration that a novel SCPL protein from Arabidopsis is required for sinapoylation and benzooylation of glucosinolates in seeds. Further, we have found that over-expression of an SCPL protein that synthesizes a candidate LMM in Arabidopsis leads to significant perturbations in soluble and wall-bound phenylpropanoid content.

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 is to learn how to fundamentally alter the chemistry of the lignin polymer in such a way that it radically alters the ease with which it can be removed from biofuel crops, thus substantially increasing yields of cell wall-derived sugars and the efficiency of their subsequent fermentation without compromising the viability of the plants themselves.

Our approach to the assembly of our LMM enzymatic toolkit is based upon several attributes that these enzymes have in common. First, 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. Second, although some of these enzymes have been purified and characterized through conventional biochemical approaches, a common feature of all three protein families is that the genes that encode them can be identified by their sequence alone. Furthermore, in many cases, important inferences with regard to the function of unknown proteins can be drawn from previous phylogenetic analyses, thus allowing us to retrieve candidate genes for LMM-synthesizing enzymes from genomic and EST databases. Third, we have developed methods for the heterologous expression of these unknown proteins and robust assays with which they can be challenged with a spectrum of metabolites in a high throughput manner such that their substrate specificity, and suitability for LMM synthesis, can be determined. Finally, many proteins within each class of catalyst share enough sequence homology that we can apply our newly-developed strategies for the generation of chimeric proteins so that the proteins we identify can be further refined for the production of optimal LMMs.

When these native or engineered genes are over-expressed in biofuel crops, we expect that the phenolic nature of the LMMs will lead to their incorporation into the growing lignin polymer 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]

As recently as 15 years ago, it was generally believed that lignin monomer composition was fixed. The scientific underpinning of this viewpoint was rapidly eroded by the identification of the *fah1* mutant of Arabidopsis, the lignin of which lacked syringyl monomers [1], the characterization of maize and pine mutants that deposited lignins containing 5-hydroxyguaiacyl and dihydroconiferyl alcohol monomers, respectively [9-10], the metabolic engineering of Arabidopsis, tobacco, and poplar lines that produce lignins derived almost solely from syringyl units [2-3], and the identification of 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.

Lignin is recalcitrant to chemical and biological degradation and interferes with utilization of lignocellulosic feedstocks in biofuel production. At the same time, lignification of at least some cells and tissues is essential for plant viability. Thus, it seems clear that innovative approaches will be required to minimize the impact of lignin on biofuel production while simultaneously maintaining overall plant fitness. 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

This project has four major objectives that all focus upon the assembly and use of a toolbox for lignin modification. Our highest priority objectives have been to clone and express P450, BAHD, and SCPL genes and to characterize their encoded proteins with regard to substrate specificity employing roboticized protein expression instrumentation coupled with activity assays that use a high throughput MS-based product analysis platform. We predict that a subset of those proteins identified in these high throughput screens will generate lignin monomers with novel hydroxylation patterns or will generate dimeric phenolic LMMs. In parallel, we are using novel gene chimera construction software and a robotic gene re-assortment and reassembly method to generate synthetic P450, BAHD and SCPL proteins that produce novel LMMs not found in Nature. This approach will also generate fundamental new knowledge with regard to structure-function relationships within each class of these important catalysts.

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) lines were identified for *SCPL5*, *SCPL7*, *SCPL17*, *SCPL19*, *SCPL21*, and RNAi lines were generated for *SCPL5* and *SCPL20*. Among single KO or RNAi seeds, only *scpl17* KO and *scpl19* KO seeds showed changes in putatively sinapoylated compounds in total seed metabolite analyses.

Seeds of Arabidopsis have been reported to contain sinapoylated glucosinolates (GSLs), which are thought to be synthesized from hydroxylated GSLs through the action of acyltransferase(s). Based on the hypothesis that this reaction will be mediated by an SCPL protein, we used LC-MS to compare the GSL profile of wild-type and *scpl17* KO seeds. A number of putative sinapoylated GSLs were found in wild-type seed extracts, and all of the compounds were absent or decreased in the mutant seeds. As a genetic approach to confirm that these GSLs are sinapoylated, we analyzed GSLs in *fah1* and *brt1* mutant seeds because FAH1 and BRT1 are involved in synthesizing sinapoylglucose, the acyl donor used by all characterized Arabidopsis SCPL enzymes. All putative sinapoylated GSLs affected in *scpl17* seeds were also missing or absent in extracts of both *fah1* and *brt1* seeds, suggesting that SCPL17 uses sinapoylglucose as a substrate to generate sinapoylated GSLs.

Surprisingly, further GSL analyses showed that putative benzoylated GSLs were also absent in *scpl17* mutant seeds. Furthermore, we have recently found that in *sng2* (*scpl19*) mutant seeds, both sinapoylated and benzoylated GSLs are also absent or decreased, even though the only current role known for SCPL19 is in the synthesis of sinapoylcholine. 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.

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 these experiments, significant DSG accumulation was observed in the leaves of plants overexpressing *SST* and cell wall-bound sinapic acid also increased, suggesting that DSG could be transported and integrated into the cell wall.

BAHD acyltransferase proteins

As a first step to identify and clone candidate BAHD acyltransferases for lignin modification, we generated a phylogenetic tree including all BAHD acyltransferases identified by BLAST searching the genomes of *Arabidopsis*, *Selaginella moellendorffii*, and *Physcomitrella patens*, as well as a large number of characterized or annotated BAHD acyltransferases from other species. The BAHD family has been previously grouped into several distinct clades [14] and there appears to be a general relationship between phylogeny and substrate specificity within this family. For example, many characterized genes belonging to Clade V have been shown to encode enzymes which take hydroxycinnamic or benzoic acid derivatives as acyl donors, making this clade attractive for our purposes of identifying enzymes capable of producing LMMs. On the basis of these observations, we are currently developing a degenerate PCR-based approach for identification and cloning of Clade V BAHD acyltransferases from plant species of interest (e.g. those known to synthesize candidate LMMs). Using our sequence alignments, we selected three conserved sequence motifs to design a set of semi-nested degenerate primers. Amplification of genomic DNA from relevant species with these primers yielded a large number of PCR products, which are currently in the process of being cloned and sequenced.

The second main focus of our work to date has been the development and refinement of an assay for use in determining substrate specificity of candidate acyltransferases, cloned using either the degenerate PCR approach described above or more directed cloning strategies. By definition, acyltransferases take two substrates (an acyl donor and an acyl acceptor), the correct identification of which is required to demonstrate enzyme activity. This presents a significant challenge to any *a priori* screen for substrate specificity of this class of enzymes. Increasing the difficulty of this problem in our specific case is the fact that the acyl donor substrate taken by BAHD acyltransferases is a CoA thioester, which in the great majority of cases are not commercially available. Despite these challenges, we have developed a BAHD acyltransferase assay which is facile, robust, and scalable to high throughput. First, we have decided at least initially to limit our search to those BAHD acyltransferases that utilize either a benzoyl or a hydroxycinnamoyl CoA thioester as an acyl donor. This strategy both greatly reduces the number of possible combinations of substrates to be tested, as well as increasing the probability that a hit in our screen will yield a potential LMM-producing enzyme. In our assay to test whether a candidate BAHD acyltransferase is capable of utilizing a given CoA thioester and a given acyl acceptor, we combine the corresponding free acid of the CoA thioester, the acyl acceptor, ATP, CoA, recombinant CoA ligase (*Arabidopsis* *4CL1*, *4CL4*, or *BZO1*, depending on the acyl donor), and the candidate BAHD acyltransferase. We then detect acyltransferase activity either by monitoring reaction products by HPLC,

or by monitoring CoA release using DTNB (5,5'-dithiobis-2-nitrobenzoic acid; Ellman's reagent), which yields a colored product upon reacting with free thiols. Crucially, this reaction can be carried out at room temperature, in a single tube (or well), without the need for purification of either the CoA ligase or the BAHD acyltransferase in question. Furthermore, the use of DTNB allows reaction progress to be monitored in real time in a large number of parallel reactions, making this assay ideal for high-throughput volume and kinetic analysis.

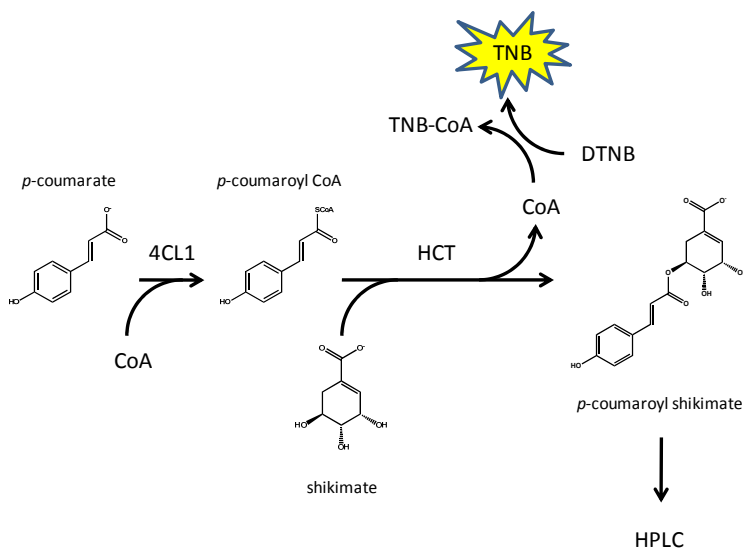


Figure 1: Scheme of the coupled CoA ligase/acyltransferase assay. Acyltransferase activity is detected as released free CoA reacts with DTNB to yield TNB, which has a characteristic absorbance at 420nm. Enzyme catalyzed hydrolysis of the CoA thioester in the absence of acyl acceptor is slow, but measurable, and is taken into account when measuring acyltransferase activity. Identity of reaction product(s) can be confirmed by HPLC analysis.

Cytochrome P450-dependent monooxygenases

P450s are a diverse superfamily of heme-thiolate proteins. Usually they are a part of multicomponent electron chains in enzymatic reactions like NADPH- and O₂-dependent hydroxylation reactions. The analysis of a number of mutants and transgenic plants has demonstrated the role that P450s play in determining lignin monomer composition.[1-4] We predict that the isolation of additional P450s will enable us to generate lignin precursors with novel hydroxylation patterns which will in turn generate lignins with novel chemical properties that may improve lignin degradability.

To identify P450s that act against phenylpropanoids or use phenylpropanoid-like substrates, we have heterologously expressed and analyzed a panel of 25 different Arabidopsis P450s. Genes of interest were identified using phylogenetic and expression analysis databases (using BAR Expression Angler at www.bar.utoronto.ca). Based on the co-expression of P450s with other small molecule metabolic enzymes such as glycosyltransferases, candidate genes were selected mainly from the 71, 76, 81 and 94 P450 families. For our initial activity studies aimed at substrate identification, we have employed a yeast expression system using Gateway technology in pYEDP60 and

transformed into the WAT11 strain of the yeast *Saccharomyces cerevisiae*. To date, approximately 600 assays have been performed with the 25 expressed P450s in the presence of substrates from the coumarin and flavonoid families, but no novel activities have been detected.

In parallel, we are screening for biochemical phenotypes in T-DNA insertion mutants defective in P450 genes of interest. The high expression of the cytochrome P450 81F4 (At4g37410) in the roots of *Arabidopsis* prompted us to analyze methanolic root extracts of a CYP81F4 KO line, and this experiment revealed that two major UV-absorbent peaks present in wild-type roots are absent in the mutant. Instead, three other compounds are detectable in mutant extracts, with retention times of 4, 7.7 and 16 minutes, which are likely to include the substrate for CYP81F4 or subsequent metabolites. LC-MS characterization of some of these compounds has provided initial clues as to their identity.

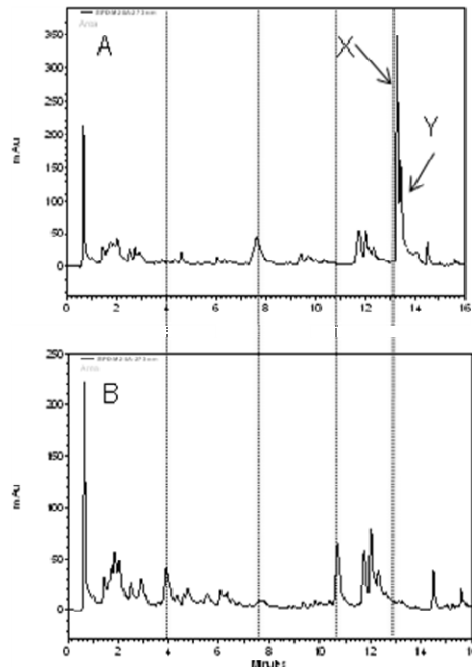


Figure 2: HPLC analysis of root extracts of 28-day-old wild-type *Arabidopsis* (A) in comparison to the root extracts of T-DNA Salk KO line of CYP81F4 (B). CYP81F4 appears to be required for the biosynthesis of UV-absorbent compounds X and Y. Novel compounds with retention times of 4, 7.7 and 16 minutes accumulate in the mutant.

Bioinformatic analyses of phenylpropanoid cytochrome P450s

To guide the formation of chimeras between P450s of phenylpropanoid metabolism and provide a basis for analyzing our experimental results we have built an internal, curated database of 500 biosynthetic P450s from plants. While other databases are available, we thought it critical to have our own in order to accurately organize the database by likely substrate specificity (which has not been experimentally determined for most of these sequences).

This collection of sequences was used for the generation of multiple sequence alignments to reveal the modules of sequence that can be swapped during chimera formation. Aware that this is a critical step (incorrect alignments will lead to swapping of non-interchangeable sequences) we have tested multiple sequence alignment programs (MUSCLE, T-Coffee, and CLUSTALW) on these sequences and determined that the alignment program MUSCLE gave superior results on such a diverse set of proteins. The MUSCLE alignment showed significantly fewer introduced gaps that appeared artifactual, as judged by the occurrence of nearby compensating gaps in other sequences. Nonetheless, the MUSCLE alignment showed some regions that were questionable as judged visually, and we have improved the automated alignment by manual editing.

The final multiple sequence alignment was then inspected visually for suitable chimera breakpoint locations, focusing on regions surrounding the known substrate recognition sequences. Chimera breakpoint locations were chosen based on the expected conservation of environment (e.g. polar versus non-polar, small versus large side chain) for the residues on either side of the breakpoint in all the P450 classes. Such breakpoints should represent equivalent structural positions in these P450's. Approximately 10 such sites have been identified. A subset of them will be employed in our first sets of site-directed chimeras.

We have begun to develop high-throughput *in vivo* assays for the P450 enzymes that are involved in phenylpropanoid metabolism derived from *Arabidopsis thaliana* (C4H, F5H, C3'H) and *Selaginella moellendorffii* (C4H and F5H). Each of these assays are conducted in heterologous WAT11 yeast strains that also express the corresponding P450 reductase from *Arabidopsis*. Two general approaches to high-throughput assays are being pursued.

The first approach is a high-throughput version of a previously-established HPLC-based assay that has been used extensively in our lab. The original assay has been modified and stream-lined for high-throughput implementation. A seven minute chromatographic separation has been developed, suitable for the identification of reaction products from chimeras with known or novel activities. Although HPLC-based detection must be carried out individually (or in small pools) and is thus inherently limited in throughput, it has the advantage that novel enzymes with altered hydroxylation patterns can be identified and that compounds produced in small amounts by enzymes with low activity can be detected. We are now in the process of validating this method to ensure its reproducibility, robustness, and sensitivity in the large-scale assay of the activity of more than a thousand potential chimeric protein variants that we expect to generate.

For more rapid screening of a large number of chimeric proteins, we have begun to test the feasibility of using differential UV absorbance of hydroxylation substrates and products in a multi-well plate reader. Absorbance measurements of the *in vivo* assay products (with or without the addition of a rapid purification step) are being tested. This method will allow us to quickly screen thousands of recombinant proteins and allow us to identify active proteins for further testing.

As a control and reference for the proposed site-directed recombination methods, we have conducted initial trials implementing a random chimera generating approach (gene shuffling) for recombining two divergent C4H genes from *Arabidopsis thaliana* and *Selaginella moellendorffii*. The CLERY method that we are using combines *in vitro* shuffling with *in vivo* recombination and has previously generated active human-derived P450 chimeras.[15] Our initial experiments have produced a number of candidate shuffled genes. We are currently analyzing some of the candidates to determine the frequency of recombination and then to evaluate their catalytic capacity.

Conclusions

In this progress report, we have outlined our initial efforts towards isolating genes of potential value in the production of LMMs, lignin building blocks which we anticipate will improve the degradability of lignin while leaving intact its critical function in the cell wall. Our work has focused on three classes of enzymes: cytochrome P450s, SCPL acyltransferases, and BAHD acyltransferases. Specifically, we have taken major steps towards the development of an *in vitro* mutagenesis approach for the generation of novel P450s to modify hydroxylation patterns in lignin, and have begun to explore the function of novel P450s in Arabidopsis metabolism. In the case of the SCPL acyltransferases, we are attempting to gain insight into the structure/function relationships of this family of enzymes to facilitate prediction of potential LMM-producing enzymes. Finally, we have developed an easily scalable assay for high-throughput BAHD acyltransferase substrate determination. These preliminary results represent essential first steps toward the identification of candidate genes that produce LMMs. It is our hope that when these native or engineered genes are over-expressed in biofuel crops, the phenolic nature of the LMMs produced will lead to their incorporation into the growing lignin polymer. If such experiments come to fruition, we envision a future in which LMM-containing crops could vastly increase the efficiency of cellulosic biofuel production and dramatically curb our excessive emission of greenhouse gasses.

Publications

1. None to date

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