Assembly of a lignin modification toolbox

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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, or engineering of, additional P450s that lead to unique hydroxylation patterns in monolignols will generate lignins with novel chemical properties that may improve lignin degradability. We are currently evaluating the metabolic profiles of Arabidopsis lines in which the genes encoding P450s have been inactivated. Using this approach, we have identified a P450 that is involved in the synthesis of a hydroxylated glucosinolate accumulated in roots, and another that is required for the synthesis of a novel phenolic secondary metabolite accumulated in stems. In a parallel approach, we have generated a large family of chimeric P450 proteins by recombining cinnamate 4-hydroxylase genes from two distantly related plant species. Many of these proteins retain 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.

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 successfully employed a degenerate PCR-based approach using conserved amino acid sequences from BAHD family members to obtain partial sequences of seven novel acyltransferase-encoding genes from the Asian fiber crop kenaf (*Hibiscus cannabinus*), which is known to accumulate the candidate LMM coniferyl ferulate.

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. During the past year, 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.

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. We predict that a subset of those proteins identified in these 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 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

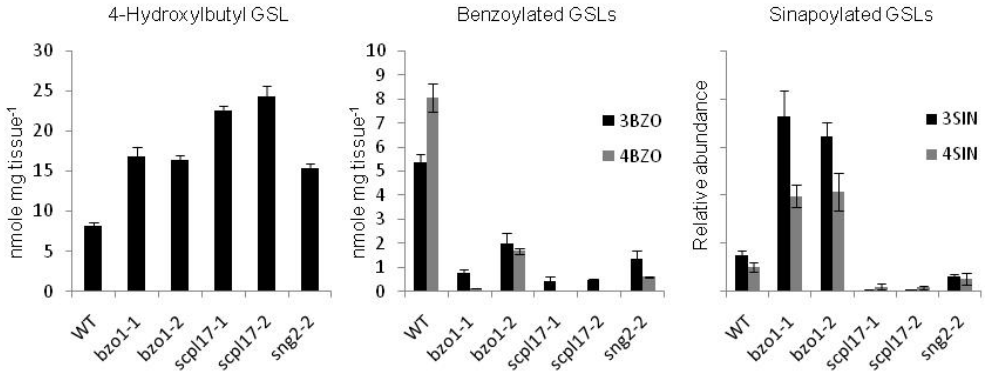

Figure 1: Glucosinolate analyses in *scpl17*, *scpl17*, and *sng2* mutant seeds. Both benzoylated and sinapoylated glucosinolates were dramatically reduced in *scpl17* and *sng2* mutants while 4-hydroxybutyl glucosinolate increased. Error bars indicate standard deviations of four replicates.

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. Further characterization of these enzymes following heterologous expression is ongoing.

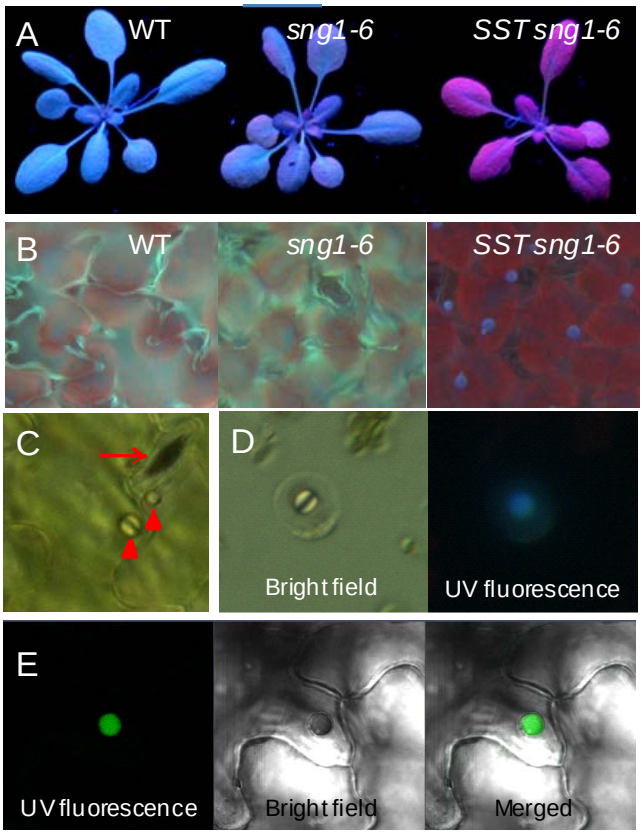

Figure 2: Characterization of SST overexpressing SMT-deficient plants. (A) UV fluorescence of 3-week old plants. (B) UV fluorescence of the 7th leaves of 3-week old plants under microscope. (C) Adaxial epidermis of a *SST sng1-6* leaf. The arrow indicates a stomata. Two arrowheads indicate the putative 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.

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, there was virtually no increase in the amount of DSG accumulated, probably because of the competing activity of sinapoylglucose:malate sinapoyltransferase (SMT), which uses the 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.

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.

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.[14] 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. To attempt to identify enzymes of this type, we are currently working to clone BAHD acyltransferases from plant species that either are known to synthesize promising LMM candidates or for which the full genome sequence is available. In parallel, we have developed and successfully scaled to high throughput an acyltransferase assay for biochemically screening the activity of these enzymes towards a range of potential substrates.

We have successfully employed a degenerate PCR-based approach using conserved amino acid sequences from BAHD family members to obtain partial sequences of seven novel acyltransferase-encoding genes from the Asian fiber crop kenaf (*Hibiscus cannabinus*), which is known to accumulate the candidate LMM coniferyl ferulate. Transcripts of two of these genes have been detected in tissue surrounding the core of the kenaf stem, where coniferyl ferulate is synthesized *in vivo*. Phylogenetic analysis strongly suggests that one of these genes encodes hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT), an enzyme of the core phenylpropanoid pathway, while the closest relatives of the other novel acyltransferase are involved in the synthesis of benzyl benzoate or phenethyl benzoate, which are strikingly similar in structure to conferyl ferulate. Currently, RACE (rapid amplification of cDNA ends) is being used to clone the 5' and 3' regions of these genes. Once full length sequences have been obtained, they will be expressed in *E.coli* and tested for activity against candidate substrates using the assay described below.

It was our first goal to develop a facile, scalable assay with which to screen for acyltransferase activity. The assay we have developed allows all of the reagents necessary for the synthesis of the CoA thioester, as well as the acyltransferase reaction, to be combined into one tube or well, and the reaction carried out at room temperature. Reaction products can then be monitored by HPLC or in real time using DTNB (5,5´-dithiobis-2-nitrobenzoic acid; Ellman's reagent) to detect CoA release from the acyl donor. Currently, we are performing this assay in 96-well plates in which we can test the activity of a particular BAHD acyltransferase towards over 60 candidate acyl acceptors simultaneously. We are currently focusing on only benzoyl CoA or hydroxycinnamoyl CoA as acyl donors in order to greatly reduce the number of substrate combinations to be tested. So far, the assay has shown to be very reliable on proteins of known function (such as HCT; Fig. 3) and is currently being tested on uncharacterized acyltransferases of unknown function which have been cloned from Arabidopsis and the recently sequenced

lycophyte *Selaginella moellendorffii*, a member of a group of plants which last shared a common ancestor with flowering plants like Arabidopsis approximately 400 million years ago. While this initial screening for substrate specificity is not exhaustive, positive hits for activity towards a given substrate(s) gives us a direction to follow with further biochemical and genetic analysis.

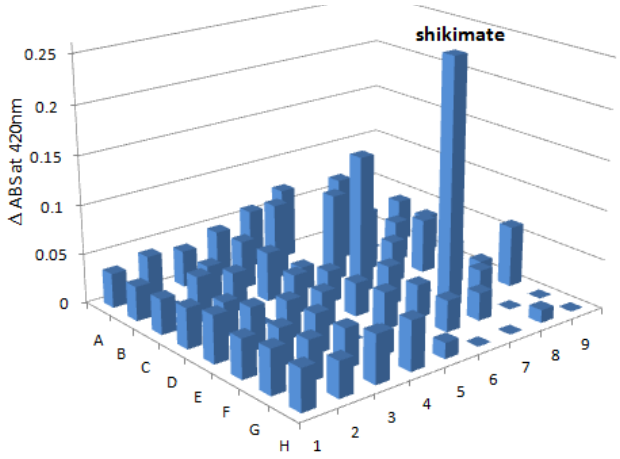

Figure 3: Proof of principle of high throughput acyltransferase screen. Using *p*-coumaroyl CoA as the acyl donor, *Selaginella* HCT was tested for acyl transferase activity towards 72 different acyl acceptors (all present at a concentration of 10 mM) in a 96-well plate. Each bar represents a well in the reaction plate, with the height indicating the total change in absorbance at 420nm after one hour. (Blank positions showed some absorbance but represent enzyme-independent reactions.) The position of shikimate, the likely *in vivo* substrate of Selaginella HCT, is indicated.

Cytochrome P450-dependent monooxygenases

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 with as-yet-unidentified functions, we screened for biochemical phenotypes in T-DNA insertion mutants defective in P450 genes with unique expression profiles. 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. While HPLC analysis showed the accumulation of 1-methoxy indol-3-ylacetonitrile (1MOIAN) in the wild type, this compound was absent in the mutant and indol-3-ylacetonitrile (IAN) accumulated in its place. It is known that IAN and its methoxylated derivatives are the break-down products of indole-3-yl-methyl glucosinolates (I3Ms) the decomposition of which is initiated by the plant enzyme myrosinase (a type of β-thioglucoside glucohydrolase). Consistent with this suggestion of the origin of 1MOIAN, we could show that wild-type Arabidopsis contains indole-3-yl-methyl (I3M), 1-methoxy-indole-3-yl-methyl (1MO-I3M), and 4-methoxy-indole-3-yl-methyl (4MOI3M)

glucosinolate while IMOI3M was absent in the mutant and its precursors I3M accumulated to higher levels. These data suggest that I3M or one of its precursors is the substrate for CYP81F4 and that the enzyme is a novel N-hydroxylase. Although this enzyme is unlikely to be useful directly in the generation of LMMs, the identification of its function is proof of principle of our approach to P450 characterization, and given the relative dearth of information concerning structure-function relationships in plant P450s, the elucidation of its function will still inform our other P450-related goals.

Using a similar approach, we have recently determined the function of a paralog of ferulate 5-hydroxylase in Arabidopsis. Although over 55% identical to one another, ferulate 5-hydroxylase uses coniferaldehyde and coniferyl alcohol as substrates; whereas, its paralog is a *p*-coumaraldehyde 3-hydroxylase required for the synthesis of a new class of phenylpropanoid small molecules we have named arabidopyrones. This new enzyme will be an immediate and valuable addition to the set of phenylpropanoid hydroxylases we will use in the chimerigenesis experiments described below.

Chimeragensis and high throughput analyses of phenylpropanoid cytochrome P450s

We have set up a high-throughput platform with which to screen heterologous yeast strains expressing *Arabidopsis thaliana* and *Selaginella moellendorffii*-derived cytochrome P450 monooxygenases. These enzymes are instrumental in phenylpropanoid metabolism, the major metabolic pathway involved in the production of lignin precursor molecules. To engineer novel P450 enzymes with unique hydroxylation patterns, we are employing a site-directed recombination approach that generates chimeric proteins with modified structural/functional characteristics of both parental genes. Chimera breakpoint locations were chosen based on the conservation of amino acid residue characteristics (e.g. polar versus non-polar, small versus large side chain) for the residues on either side of the breakpoint using a multiple sequence alignment of more than 500 plant-derived P450 enzymes.

Using a primer extension PCR strategy we generated 64 chimeric proteins originating from the *Arabidopsis* and *Selaginella* cinnamate 4-hydroxylase coding regions with five breakpoints. This strategy takes advantage of hybrid primers designed to contain both parental gene sequences upstream and downstream of suitable chimera breakpoint locations, focusing on regions surrounding substrate recognition sequences. This PCR-based primer extension method is currently being developed for high-throughput implementation using robotic manipulation and automation in a multi-well plate format.

We are currently using the same robotic system to develop the high-throughput *in vivo* heterologous yeast assays. As a pilot study, we characterized the 64 *Arabidopsis/Selaginella* cinnamate 4-hydroxylase chimeras that were produced. Over 50% of these chimeric proteins were enzymatically active, despite the differences in amino acid sequences between their parents.

Progress

In this progress report, we have outlined our 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 isolated and characterized two P450s whose functions had not previously been known. 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, and are directly testing the impact of the overexpression of one related to LMM production. 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 gases.

Future Plans

Our work on SCPL and BAHD acyltransferases is in its early days, but is proceeding on target. This work will continue and we expect it to identify proteins of use in the production of LMMs. Based upon the success of our initial studies, we are now preparing to generate chimeric P450 proteins derived from lignin biosynthetic P450s each of which varies subtly in substrate character and hydroxylation regiospecificity. These will include cinnamate 4-hydroxylase, *p*-coumaroylshikimate 3'-hydroxylase, ferulate 5-hydroxylase, and the *p*-coumaryladehyde 3'-hydroxylase from *Arabidopsis thaliana* involved in arabidopyrone synthes (F5H2). We will also include in the chimerigenesis set a novel *p*-coumaraldehyde/*p*-coumaryl alcohol 3,5-hydroxylase we recently identified from *Selaginella moellendorffii* (SmF5H).[15] This selection of enzymes uses phenylpropanoid acids, aldehydes and alcohols as substrates and hydoxylates them in the 3, 4, or 5 position (Fig. 4). By recombining these enzymes, we expect to be able to generate new catalysts for the redirection of phenylpropanoid flux in plants.

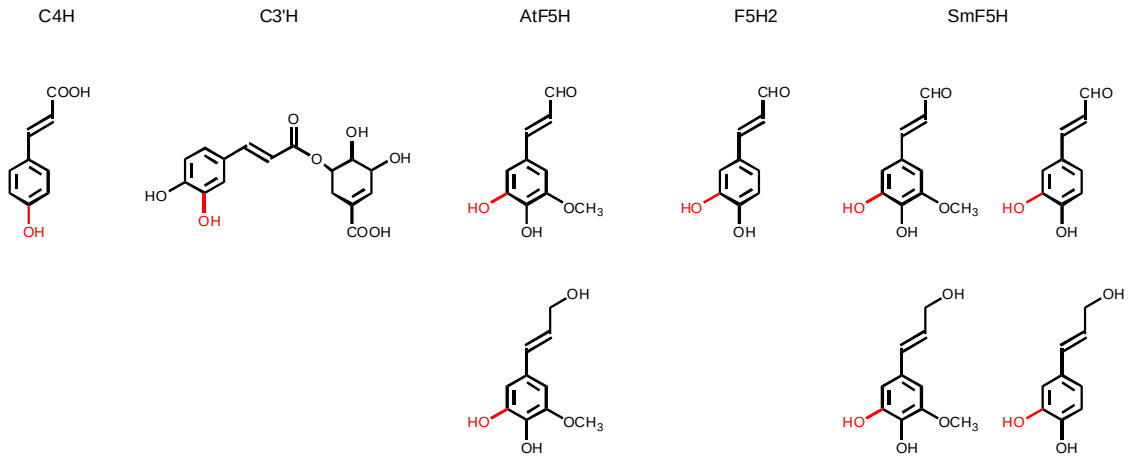

Figure 4: Substrates of P450s to be used to make chimeras in this project. The position of hydroxylations catalyzed by these P450s is shown with a red OH group.

Publications

1. Invited seminar,Yale University, New Haven CT, April 2010. Lignification of plant cell walls: a milestone in plant evolution.
2. Invited seminar, University of Massachusetts – Amherst, March 2010. Lignification of plant cell walls: a milestone in plant evolution.
3. Invited seminar, Global Climate and Energy Project, Stanford University, October 2009. Assembly of a lignin modification toolbox.
4. Invited seminar, University of British Columbia, Vancouver BC, May 2009. Lignification of plant cell walls: a milestone in plant evolution.
5. Invited seminar, University of Minnesota, Minneapolis MN, April 2009. Genetics and biochemistry of lignin biosynthesis in land plants.

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