Efficient Biomass Conversion:
Delineating the Best Lignin Monomer-Substitutes

Final Report

Investigators

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Abstract

[This project continued through 2012 in a no-cost extension mode; although publications and presentations continue from material under it, we are considering this as a final report. We shall nevertheless continue to update GCEP with publications resulting from it.]

The three year plan was to delineate a set of approaches for successfully altering lignin structure, in a way that allows plant cell wall breakdown to produce biofuels in a more energy-efficient manner, by providing alternative plant-compatible monomers to the lignification process. In fact, the bold plan for much of the work under the GCEP 2.3 "Renewable Energy Biomass" program is ***to 'redesign' lignin to be chemically labile*** (but obviously not biologically labile) so that the processing costs are vastly reduced and the net energy balance of biomass conversion is significantly higher.

During the research, a set of potential monolignol replacements was synthesized (or otherwise obtained) and all compounds were subjected to a set of tests to determine if they had the necessary attributes to provide valuable traits to biomass crop plants. The tests ranged from determination of their compatibility with lignification through to the enhanced digestibility of lignified walls (in which the new monomers were incorporated) in ruminant systems and to saccharification with various pretreatments.

Rosmarinic acid was found to be a particularly promising lignin monomer-replacement. Work was completed and published, garnering cover-article status in ChemSusChem.[1] This monomer contains caffeyl units and caffeates that are in fact very compatible, incorporating rosmarinic acid beautifully into lignins, and introducing a readily cleavable ester linkage into the backbone of the lignin such that depolymerization is spectacularly more facile. Rosmarinic acid strikingly enhanced alkaline lignin extractability and promoted subsequent cell wall saccharification by fungal enzymes. Interestingly, incorporating rosmarinic acid also improved cell wall saccharification by fungal enzymes and by rumen microflora even without alkaline pretreatments, possibly by modulating lignin hydrophobicity and/or limiting cell wall cross-linking.

Introducing various catechins and gallates (from plant tannin pathways) into lignification produces quite remarkable improvements in degradability;[2-4] the improvement is related quite strikingly to the phenolic-OH content of the monomer-substitute. Our paper on "Identifying new lignin bioengineering targets: Impact of epicatechin, quercetin glycoside, and gallate derivatives on the lignification and fermentation of maize cell walls"[2] was the winner of the *Journal of Agriculture and Food Chemistry*'s 2013 Research Article of the Year Award (to John Grabber). A patent has been filed.

In collaboration with Boerjan's group, we tested a new multi-misregulation approach to produce the first transgenics that derive their lignins primarily from non-traditional monomers. The approach was aimed at improving digestibility by reducing the extent of cell-wall-polymer cross-linking, but the plants are not healthy, and not worthy of testing for digestibility implications. Nevertheless, the approach was informative in delineating just how far lignification can be perturbed away from normal monolignols and sheds light on how biosynthetic pathways interact. The paper appeared as a cover article in *The Plant Journal*.[5]

Integral to the work has been the development of tools useful to researchers, including synthetic schemes for producing valuable compounds involved in the pathways.[6-8] We also prepared fluorescence-tagged monolignols applicable to studying *in vitro* lignification, for imaging the cell wall, and for mechanistic studies.[9,10] In collaborations with our group, Boerjan's group has developed significant new LC-MS-based methodologies for metabolomics, allowing detailed profiling of oligolignols.[11-14]

Overall, this fundamental project, coupled with the integral projects from other GCEP research in the lignin area (by Boerjan's, Chapple's, and Halpin's groups) has now identified several promising strategies and validated several specific examples of monolignol-replacement compounds that have the potential to significantly improve the energetics of biomass conversion, as we have now reviewed.[15] All of these strategies are aimed at making the lignin less of a recalcitrance factor and, in some cases, making it significantly easier to depolymerize. The ultimate GCEP goal of reducing greenhouse gas emissions at a global scale will depend on the ability to successfully engineer such traits into biomass crops, a goal of active research worldwide at present.

Introduction

The objective of this work is to reduce the energy requirements for processing lignocellulosic materials by structurally altering lignin, by modifying its monomer complement, to allow the biomass resources to be more efficiently and sustainably utilized. Its aims were to identify lignin monomer-substitutes that are fully compatible with the polymerization processes inherent in plant lignification and that, additionally, can produce modified lignin polymers that render plant cell walls less recalcitrant toward processing to biofuels. The use of lignocellulosics for biofuels, and the improvements if feedstocks can be selected/engineered for easier processing, will contribute enormously to minimizing greenhouse gas production in the transportation fuels sector.

The approach was to synthesize and test a strategic range of novel plant compatible monomer-substitutes for their abilities to incorporate into lignins, and then to determine

how such incorporation affects biomass processing in biomimetically lignified cell wall systems. The classes of monomer substitutes include: a) Difunctional monomers or monomer conjugates linked via cleavable ester or amide (and/or hydrophilic) functionality; b) Monomers that produce novel cleavable functionality in the polymer; c) Hydrophilic monomers; d) Monomers that minimize lignin-polysaccharide cross-linking; and e) Monomers that produce simpler lignins.

The ability of a chosen monomer to incorporate into lignins (copolymerizing with the traditional monomers) is determined by *in vitro* biomimetic lignification involving the phenolic radical coupling reactions that typify the lignification process. Those that successfully make co-polymers are next polymerized into a suspension-cultured cell wall system to further delineate their polymerization efficacy and to provide biomimetic cell wall material for preliminary testing of conversion efficiency following selected pretreatments and in a variety of processes. These materials also provide the cell-wall-NMR database to allow the success of plant transformations to be determined.

Monomer-substitutes that are most promising are revealed to other plant researchers so that the process of understanding the pathways that produce the monomers, obtaining the required genes, and testing plant transformations, can proceed most expediently.

Background

Over the past decade it has become apparent that the metabolic malleability of lignification, the process of polymerization of phenolic monomers to produce lignin polymers, provides enormous potential for engineering the resistant polymer to be more amenable to processing, as reviewed.[16-20] Massive compositional changes can be realized by perturbing single genes in the monolignol pathway, particularly the hydroxylases. More strikingly, monomer substitution has been observed in the process of lignification, particularly in cases where a plant's ability to biosynthesize the usual complement of monolignols is compromised. These substitutions include products of incomplete monolignol biosynthesis such as 5-hydroxyconiferyl alcohol,[21] ferulic acid,[22] and coniferaldehyde and sinapaldehyde,[21] in some cases at quite high levels and without obvious pleiotropic effects. This suggests that lignin composition and structure can be altered, leading to plants with characteristics for improved processing to biofuels.

Replacing the entire monomer component of lignification with a novel monomer is unlikely to be an effective strategy that is "acceptable" to the growing plant. Introducing strategic monomers into the normal monolignol pool is, however, a viable proposition. To date, incorporation of up to 30% novel monomer has produced plants with no pleiotropic effects or obvious growth phenotypes. A range of alternative monomers appears to be consistent with the GCEP RFP criteria of maintaining the plant's structural and functional integrity, but any approach will require empirical testing. The key here is to home in on the best strategies for plant-compatible monomer substitution that will produce lignins that substantially ease processing of the cell wall.

Observations to date have allowed us to detail some ideal properties of monolignol substitutes.[17] When such compounds are introduced into lignins, even at significant levels, the plants show no obvious growth/development phenotype. Monomers that have accessible conjugation into the sidechain allowing for so-called "endwise" β–O–4-

coupling seem to fare the best. Examples are: 5-hydroxyconiferyl alcohol, the hydroxycinnamaldehydes, hydroxycinnamate esters, and acylated hydroxycinnamyl alcohols. Due to incompatibilities in radical coupling reactions, *p*-hydroxyphenyl moieties fare less well than guaiacyl or syringyl moieties, at least when incorporating into guaiacyl-syringyl lignins, but other phenolics have not been well studied.

Without regard to plant biochemistry, it is easy to come up with a set of weird and wonderful monomers from simple chemical principles, from chemical catalogs, or by design. At this initial stage, however, the *only* monomers in contention are those that plants can biosynthesize; i.e., for which *in planta* biosynthetic pathways (and hence enzymes and genes) exist. All of the potential lignin monomers we intend to test have been isolated from various plant materials. The derivation of some is not entirely obvious but, if plants are truly making them, then enzymes and genes for the required biochemical pathways must be in place. The classes of monomers are considered the most fruitful to explore are as described above.

Recent external developments in the field include the following (some from our own non-GCEP research)…

1. A striking example of a new type of lignin, in fact derived from 'the missing monolignol,' caffeyl alcohol, has been discovered in various seed coats.[23] In the seeds containing this 'new' caffeyl alcohol polymer, the lignin component is derived solely from caffeyl alcohol (and not the normal monolignols, coniferyl and sinapyl alcohols). The implication for this project and to GCEP is twofold. First, the monomer has a catechol (*o*-diphenol) moiety that results in internal trapping of lignin-intermediate quinone methides, meaning that this lignin will not become involved in lignin-polysaccharide cross-linking (that normally involves trapping of the quinone methide by polysaccharides); cell walls containing such lignins are therefore anticipated to be more easily and completely saccharifiable. Second, this lignin is particularly homogenous, rigid, linear, and seemingly insoluble and unswellable in aqueous solvents. As such, the polymer should be investigated for its ability to more efficiently sequester carbon.
2. Genes encoding transferases that make monolignol hydroxycinnamate conjugates have been identified. The first is a transferase implicated in attaching *p*-coumarate to monolignols in all grasses, so-called PMT (*p*-coumaroyl-CoA : monolignol transferase).[24] The second is its ferulate analogy, FMT (feruloyl-CoA : monolignol transferase), which has been described in conference talks/posters,[25-30] and is the subject of a patent application, but has not yet been published. The potential of monolignol ferulates to engineer readily cleavage ester linkages into the backbone of the lignin polymer, analogous to some approaches here, has been discussed.[31,32]
3. New dual-function hydroxylases, along with their associated *O*-methyltransferases for the monolignol pathway have been discovered in the ancient plant, selaginella.[33-35] They have already been engineered into Arabidopsis, showing that they can execute both functions and creating rather novel lignins. There is therefore potential to increase the efficiency of the monolignol pathway.
4. New transgenics are becoming available (sorry, details not provided here), in plants that are quite phenotypically normal, in which the lignins are composed almost

entirely of novel units, i.e., only low levels of the normal monolignols. Such studies continue to reveal the metabolic plasticity of the lignification process and open up new possibilities for substantially altering the composition and structure of lignins in ways that may improve plant biomass processing.

Results

In the GCEP grant application, we provided a rather extensive set of potential monomers (not shown as such here, but see Figure 1), in various classes, to consider as monolignol-substitutes to improve the processing of plant materials (by lowering the recalcitrance posed by the lignin component of their cell walls).

Figure 1 highlights the compounds we studied, providing a summary all of the alternative plant-derivable monomers that have been synthesized or acquired and tested to some degree, with that testing indicated (see Figure caption). In some cases, we are still finishing and writing up the studies. We hope that it is not unreasonable to note that such studies are rather time-consuming and were too extensive and ambitious to complete within the period of the GCEP study. Nevertheless, the research is considered to be sufficiently compelling that we are 'finding other means' to complete the work for publication and dissemination.

The progress on the syntheses and the testing, in synthetic lignins and in a plant cell wall model system, of all of these was reported in prior annual reports. Only the major findings will be noted here.

1. Rosmarinic Acid

Work on the monomer-substitute candidate rosmarinic acid was completed and published, garnering cover-article status in ChemSusChem, one of the premier green chemistry and sustainability journals.[1] [This monomer, that came up in a conversation many years ago with Chris Somerville, was regrettably not pursued immediately due, quite frankly, to a reticence (from John Ralph) about caffeyl units and caffeates in lignin! It turns out that these are in fact very compatible, and that rosmarinic acid, a structure that contains such units, incorporates beautifully into lignins, introducing a readily cleavable ester linkage into the backbone of the polymer such that depolymerization is spectacularly more facile. In a little more detail…

In *in vitro* synthetic lignin (DHP, dehydrogenation polymer) experiments, rosmarinic acid readily underwent peroxidase-catalyzed copolymerization with monolignols to form polymers with benzodioxane inter-unit linkages, suggesting that fewer lignin-carbohydrate cross-links could be formed via lignin quinone methide intermediates. Incorporation of rosmarinic acid permitted extensive depolymerization of DHPs by mild alkaline hydrolysis, via cleavage of ester linkages within the rosmarinic acid moiety (in the lignin) itself. Copolymerization of rosmarinic acid with monolignols modestly depressed lignification of cell walls, but without adversely affecting wall-bound peroxidase. Reduced formation of wall-bound lignin may be associated with the formation of fewer lignin carbohydrate cross-links or smaller readily extracted polymers. Rosmarinic acid strikingly enhanced alkaline lignin extractability and promoted

subsequent cell wall saccharification by fungal enzymes. Interestingly, incorporating rosmarinic acid also improved cell wall saccharification by fungal enzymes and by rumen microflora even without alkaline pretreatments, possibly by modulating lignin hydrophobicity and/or limiting cell wall cross-linking.

The success in the model systems suggests that this is a lignin modification pathway worth pursuing. Although the acid group in rosmarinic acid is of some concern, the beneficial effects of its incorporation, if it can be accommodated *in planta*, could rival the emerging success with another difunctional monomer, the monolignol ferulate conjugates (that predated and, unfortunately, could not be part of this GCEP project).[31,32] Rosmarinate biosynthetic pathway genes are known, and groups are attempting its introduction into the xylem-specific pathway in plants.[36-40]

2. Catechins and gallates

We published two studies on catechin derivatives.[2-4] Overall, these monolignol substitutes improved the inherent degradability of non-pretreated cell walls by restricting lignification or possibly by reducing lignin hydrophobicity or cross-linking to structural polysaccharides. For example, when epicatechin derivatives were included in the lignification, gas production via rumen microbes (a good measure of cell wall fermentability) was markedly enhanced with increasing phenolic hydroxyl content of the monomer-substitute (in a virtually linear fashion).[41] Epicatechin (EC **21**), epicatechin gallate (ECG **22**) and epigallocatechin gallate (EGCG **27**) readily polymerized with monolignols to form lignin in concentrations equal to the high lignin control. Lignin concentrations were somewhat depressed when epigallocatechin **26** and epicatechin vanillate **23** were used to lignify cell walls. Lignified cell walls were incubated *in vitro* with rumen microflora, which produce a potent array of cell wall degrading enzymes. Gas production during incubation of cell walls (positively and highly related to cell wall fermentation and utilization by rumen microflora) was considerably enhanced by most catechin derivatives, especially epicatechin gallate **22**, epigallocatechin gallate **27**, or epigallocatechin **26**. Improved fermentability with these catechin derivatives was further supported by lower levels of non-fermented polysaccharides (NP) remaining after incubation with rumen microflora and by lower gas reduction per unit lignin (GRL) and lower non-fermented polysaccharide accumulation per unit lignin (NPAL) in cell walls. Enhanced fermentability of cell walls was positively related to the degree of catechin hydroxylation.[2]

Cell wall residues collected after a weak acid or weak alkaline pretreatment were also subjected to enzymatic hydrolysis with a crude fungal cellulase and xylanase mixture. The inclusion of most catechin derivatives enhances glucose release, especially at the early stages of hydrolysis (6 h) and this was more pronounced after weak acid or base pretreatment of cell walls.

The results with catechins and gallates were sufficiently encouraging that a provisional patent was filed on “Incorporation of flavan-3-ols and gallic acid derivatives into lignin to improve biomass utilization” – this was followed up by a full patent application.

Some monolignol substitutes, chiefly readily cleaved bi-phenolic conjugates like epigallocatechin gallate **27** or diferuloyl polyol esters (such as **9** and **15**), are expected to greatly boost the enzymatic degradability of cell walls following chemical pretreatment. In ongoing work, we are characterizing the enzymatic saccharification of intact and chemically pretreated cell walls lignified by these and other monolignol substitutes to identify promising genetic engineering targets for improving plant fiber utilization. Although producing cell walls that can be saccharified without requiring chemical pretreatments remains a goal, it is likely that pretreatment will be required to allow the wall polysaccharides to be fully utilized. Thus, monomer-substitutes that produce lignins that are essentially 'designed' for simplified pretreatment, are particularly attractive targets.

Our paper on "Identifying new lignin bioengineering targets: Impact of epicatechin, quercetin glycoside, and gallate derivatives on the lignification and fermentation of maize cell walls"[2] was the winner of the Journal's 2013 Research Article of the Year Award (to John Grabber), indicating the level of interest in these GCEP-based lignin alteration methods.

3. Amides, guaiacylbutenol, monolignol glucosides, miscellaneous

Work has been completed on a series of amide compounds, including clovamide, feruloyl dopamine and feruloyl methoxytyramine, and interesting compounds that have the potential to reduce cell wall cross-linking, such as guaiacylbutenol. In general guaiacylbutenol and the difunctional amides incorporated efficiently into DHP-cell walls. Among these only clovamide substantially improved the inherent ruminal fermentability of lignified cell walls. Unfortunately none of these monomers enhanced the saccharification of lignified walls following acid or alkaline pretreatment as determined by digestibility assays. The monolignol glucosides incorporated very poorly into lignified cell walls and so we have not pursued further fermentation and saccharification with these monomers.

Although the difunctional amides are rather interesting natural plant-derived metabolites that can incorporate well into lignins, as established in synthetic lignins, they suffer from two drawbacks. The first is that it is actually surprisingly difficult to cleave the amides in an industrially relevant way. This means that conjugates that introduce amide linkages into the backbone of the polymer, in a manner analogous to the way rosmarinate introduces esters, this does not render the polymer significantly easier to depolymerize during biomass pretreatment. Secondly, compounds containing nitrogen might result in added-value degradation products from lignin, but the biosynthesis of such molecules would likely increase the need to provide plants with increased nitrogen-containing fertilizer. In some cases, greater fertilizer use might be justified but, as a general rule, this would undesirably increase the financial and environmental cost of growing biomass for biofuel production.

In summary, these are all considered monomers that are NOT useful as monolignol substitutes. Although this sounds negative, a significant aspect of this study was to identify not only monomer replacements that are promising to pursue, but also to rule out certain options before researchers embark on the arduous task of finding the genes and

suitably transforming (test) plants. These results are therefore considered to be also particularly worth reporting and conveying to the public. We still plan to write up the relatively modest work that went into these studies; the struggle is that, for the most part, Journals are not keen on reporting negative results.

4. Creation of a transgenic plants in which a monolignol-substitute is the major monomer

Creating a plant, even an unhealthy, growth-compromised one, in which the complement of normal monolignols is minor is simply an intriguing target, providing insight into just how far and in which directions lignin redesign might be contemplated. The goal here was to determine, for the first time, if we could develop plants in which the lignin approaches the state where none of its constitution derives from traditional monolignols! Our aim here was not to simply test this concept, but arose from a desire to profoundly alter the lignin composition and structure for both academic and, potentially, practical purposes.

This was first achieved via concomitant F5H-upregulation and COMT-downregulation, initially in *Arabidopsis*, in collaboration with the Belgian GCEP group lead by Boerjan.[5] Strikingly, when Arabidopsis *F5H1* is over-expressed in a *comt* mutant background, the lignin is derived from ~71% 5-hydroxyconiferyl alcohol, the monolignol-substitute. The resulting lignins contain an overwhelming proportion, ~92%, of novel benzodioxane units that are not detectable in control plants. Such a level of a single inter-unit type is unprecedented, in normal or transgenic plants, even for the predominant β-ether linkages in normal lignins. This is the first transgenic with a lignin that is comprised almost entirely from units that are undetectable in WT plants. Whereas this approach was successful in producing plants with lignin derived from primarily a novel monolignol-substitute, and whereas that lignin was quite simple and regular and, presumably, had reduced polysaccharide cross-linking, it has not resulted in plant materials that are agronomically sound. However, the work has helped delineate how various pathways interact and, again, is the first report showing that lignin can be comprised almost entirely from units that are undetectable in WT plants.

As interest in the notion of plants in which the normal monolignols are minor has grown, we are pleased to be involved in other studies in which we have now identified plant tissues in which the lignin is entirely derived from a novel monolignol. Thus seed coats of the vanilla bean and some cacti have their lignins made entirely from caffeyl alcohol, a ‘missing monolignol.’ We may have also identified seeds in which the lignin is made entirely from 5-hydroxyconiferyl alcohol, the target of the above transgenic experiments. What is drawing interest is that these lignins are essentially linear homopolymers and may therefore have interesting new use potential. Another amazing polymer, derived from a non-monolignol, has also been revealed at an extremely high level in a phenotypically normal transgenic plant; we are not yet at liberty to reveal the identity of this one. The important point is that such GCEP-based studies, and now beyond, are revealing the extent to which lignin alteration may be pushed!

5. Fluorescence-tagged Monolignols

A significant part of this research project was to provide research tools for other researchers in this area. We initially prepared a fluorescence-tagged monolignol applicable to studying in vitro lignification.[9] Such compounds have become highly requested and we have just submitted a paper describing plant studies using newly created fluorescence-tagged monolignols that follows from this considerable interest by researchers in such compounds that are useful for imaging the cell wall and for mechanistic studies.[10]

6. Synthetic Methodologies, New Compounds

In addition to the syntheses that were described in the full papers, and the synthesis of fluorescence-tagged monolignols noted above, the following describe syntheses of compounds useful to plant researchers. A paper describing a new and simpler synthesis of hydroxycinnamaldehydes was published; these compounds are intermediates in the lignin biosynthetic pathway and are useful to our project and to other researchers.[6] A paper on new syntheses of the 3-*O*-vanillate and 3-*O*-ferulate esters of (-)-epicatechin is being revised.[7] Efficient new syntheses of compounds that are implicated as important activated intermediates of various ferulates and sinapates, the 1-*O*-feruloyl and 1-*O*-sinapoyl-glucopyranoses were developed allowing gram-scale access to these compounds that were only previously available from plant isolates.[8]

7. Lignomics, and New approach to determining cross-coupling success

In collaborations with our group, Boerjan's group has developed significant new LC-MS-based methodologies for metabolomics, allowing detailed profiling of oligolignols.[11-14] Although our NMR-based methods allow us to readily detect novel monomer incorporation, the details of the important cross-coupling reactions (between the novel monomer and normal monolignols) remain somewhat elusive. MS methods can help us determine that, for example, 'a trimer comprises one novel monomer X and two coniferyl alcohol units,' thus establishing that cross-coupling has occurred. Our group is involved in preparing lower-molecular-mass synthetic lignins (DHPs) incorporating many or all of the monomers of Figure 1, for such analysis by the Boerjan group. This collaborative project will significantly enhance our knowledge of the compatibility of each of the monolignol-replacement compounds with the process of lignification *in vitro* and, by extension, *in vivo*. Papers are currently in progress.

8. Review of the Area

As a culmination of this research, we (in collaboration with our Belgian GCEP colleagues) published an expansive review on the subject of this research entitled "Metabolic engineering of novel lignin in biomass crops."[15]

Conclusions

GCEP's goal is in developing the basis for technology options that could lead to substantial reductions in emissions of greenhouse gases that result from energy use. This fundamental project, coupled with the integral projects from other GCEP research in the lignin area (by Boerjan's, Chapple's, and Halpin's groups) has now identified several promising strategies and validated several specific examples of monolignol-replacement compounds that have the potential to significantly improve the energetics of biomass conversion. All of these strategies are aimed at making the lignin less of a recalcitrance factor and, in some cases, making it significantly easier to depolymerize. As shown in this report, rosmarinic acid and various catechins are such examples, strikingly improving the ease with which lignins can be depolymerized in biomimetic cell wall systems. The ultimate GCEP goal of reducing greenhouse gas emissions at a global scale will depend on the ability to successfully engineer such traits into biomass crops, a goal of active research worldwide at present.

There are now several strategies identified to pursue (and, almost as importantly, strategies that can be essentially discarded as not being worthwhile). Along with the near carbon neutrality of utilizing plant biomass (instead of fossil fuel sources), lignin-modified plant materials have the potential to significantly ameliorate greenhouse gas emission in the transportation fuel industry globally. Researchers worldwide are using these leads to attempt to engineer superior processing traits into biomass crops.

Publications

The following (cumulative) papers, patents and presentations cite this GCEP grant. Further publications will result over the next year or so.

Refereed Publications

Y. Tobimatsu, S. Elumalai, J. H. Grabber, C. L. Davidson, X. Pan and J. Ralph. **Hydroxycinnamate conjugates as potential monolignol replacements: *in vitro* lignification and cell wall studies with rosmarinic acid.** *ChemSusChem*, 2012, 676-686 and COVER ARTICLE.

Zhu, Y., Mohammadi, A., and Ralph, J. **Facile synthesis of 4-hydroxycinnamaldehydes.** (2012) *BioEnergy Research* **5**(2), 407-411

Vanholme, R., Morreel, K., Darrah, C., Oyarce, P., Grabber, J. H., Ralph, J., and Boerjan, W. **Metabolic engineering of novel lignin in biomass crops.** (2012) *New Phytologist* **196**(4), 978-1000

Grabber, J. H., Ress, D. K., and Ralph, J. **Identifying new lignin bioengineering targets: Impact of epicatechin, quercetin glycoside, and gallate derivatives on the lignification and fermentation of maize cell walls.** (2012) *J. Agr. Food Chem.* **60**(20), 5152-5160

Elumalai, S., Tobimatsu, Y., Grabber, J. H., Pan, X., and Ralph, J. **Epigallocatechin gallate incorporation into lignin enhances the alkaline delignification and enzymatic saccharification of cell walls.** (2012) *Biotechnol Biofuels* **5**, 59

Y. Tobimatsu, C. L. Davidson, J. H. Grabber and J. Ralph. **Fluorescence-tagged monolignols: Synthesis and application to studying *in vitro* lignification.** *Biomacromolecules*, 2011, **12**, 1752-1761.

Y. Zhu and J. Ralph. **Stereoselective synthesis of 1-O-β-feruloyl and 1-O-β-sinapoyl glucopyranoses.** *Tetrahedron Letters*, 2011, **52**, 3729-3731.

R. Vanholme, J. Ralph, T. Akiyama, F. Lu, J. Rencoret Pazo, J. Christensen, A. Rohde, K. Morreel, R. DeRycke, H. Kim, B. Van Reusel and W. Boerjan. **Engineering traditional monolignols out of lignins by concomitant up-regulation *F5H1* and down-regulation of *COMT* in Arabidopsis.** *The Plant Journal*, 2010, **64**, 885-897 and COVER ARTICLE.

R. Vanholme, K. Morreel, J. Ralph and W. Boerjan. **Lignin biosynthesis and structure.** *Plant Physiology*, 2010, **153**, 895-905.

F. van Parijs, K. Morreel, J. Ralph, W. Boerjan and R. M. H. Merks. **Modeling lignin polymerization. I. Simulation model of dehydrogenation polymers.** *Plant Physiology*, 2010, **153**, 1332-1344.

K. Morreel, O. Dima, H. Kim, F. Lu, C. Niculaes, R. Vanholme, R. Dauwe, G. Goeminne, D. Inzé, E. Messens, J. Ralph and W. Boerjan. **Mass-spectrometry-based sequencing of lignin oligomers.** *Plant Physiology*, 2010, **153**, 1464-1478.

J. H. Grabber, P. F. Schatz, H. Kim, F. Lu and J. Ralph. **Identifying new lignin bioengineering targets: 1. Monolignol substitute impacts on lignin formation and cell wall fermentability.** *BMC Plant Biology*, 2010, **10**, 1-13.

Publications submitted

Liu, B., Butenko, M. A., Shi, C.-L., Bolivar, J. L., Winge, P., Stenvik, G.-E., Vie, A. K., Leslie, M. E., Brembu, T., Kristiansen, W., Bones, A. M., Patterson, S. E., Liljegrens, S. J., and Aalen, R. B. ***NEVERSHED* and *INFLORESCENCE DEFICIENT IN ABSCISSION* are differentially required for cell expansion and cell separation during floral organ abscission in *Arabidopsis thaliana*.** (2013) *J Exp Bot*, in review.

Ress, D. K.; Ralph, J., **Synthesis of 3-*O*-vanillate and 3-*O*-ferulate esters of (-)-epicatechin.** *Journal of Agricultural and Food Chemistry*, in revision.

Patent Applications

1. J. H. Grabber, J. Ralph. Incorporation of flavan-3-ols and gallic acid derivatives into lignin to improve biomass utilization. USA Provisional Patent, #61/325,695, April 19, 2010.
2. The above has been converted to a full Patent Application – Application Serial No.: 13/090,206; Date Filed: April 19, 2011.

Presentations

Note: This is not necessarily complete as we no longer keep a good record of presentations – there are just too many!

Y. Zhu, Y. Tobimatsu, H. Kim, C. Davidson, J. H. Grabber and J. Ralph. Identifying new lignin bioengineering targets: Hydrophilic monolignol surrogates. 243rd National Meeting of American Chemical Society, San Diego, CA, 2012.

Yang, F., Mitra, P., Zheng, K., Sun, L., Tobimatsu, Y., Smith, M., Kim, J. S., Eudes, A., Varanasi, P., Benke, P. I., Mukhopadhyay, A., Singh, S., Ralph, J., Simmons, B. A., Keasling, J. D., and Loque, D. (2012) identification and characterization of transporter mediating monolignol export in Arabidopsis. In. *Plant Biology 2012*, American Society of Plant Biologists, Austin, TX

Tobimatsu, Y., Wagner, A., Donaldson, L., Mitra, P., Loque, D., Dima, O., Niculaes, C., Boerjan, W., Grabber, J. H., and Ralph, J. (2012) Fluorescence-tagged monolignols for delineating plant cell wall lignification: design, synthesis, and preliminary applications. In. *Plant Biology 2012*, American Society of Plant Biologists, Austin, TX

Tobimatsu, Y., Wagner, A., Donaldson, L., Mitra, P., Loque, D., Dima, O., Niculaes, C., Boerjan, W., Grabber, J. H., and Ralph, J. (2012) Fluorescence-tagged monolignols for delineating plant cell wall lignification: design, synthesis, and preliminary applications. In. *2012 Gordon Conference on Plant Cell Walls*, GRC, Colby College, Waterville, ME

Y. Tobimatsu, J. H. Grabber, P. Mitra, A. Wagner, L. Donaldson, C. L. Davidson, D. Loque and J. Ralph. Fluorescence-tagged monolignols: New approaches to the study of cell wall lignification. 243rd National Meeting of American Chemical Society, San Diego, CA, 2012.

Tobimatsu, Y., Wagner, A., Donaldson, L., Mitra, P., Loque, D., Dima, O., Niculaes, C., Boerjan, W., Kim, J.-I., Anderson, N. A., Chapple, C., Zhao, J., Dixon, R. A., Bolivar, J., Fabel, T., Patterson, S., Grabber, J. H., and Ralph, J. (2012) Fluorescence-tagged monolignols for delineating plant cell wall lignification. In. *2012 GLBRC Science Retreat*, Naperville, IL

Rencoret, J., and Ralph, J. (2012) Altering plant lignins for improved biomass processing. In. *International Symposium on "Paradigms for the Pulp and Paper Industry in the XXI Century: Opportunities for a Sustainable Future"*, Seville, Spain

Ralph, J. (2012) 'New' genes from novel plants for altering lignification. In. *INRA-Versailles Plant Seminar Series*, INRA-Versailles, Paris, France

Ralph, J. (2012) 'New' genes from novel plants for altering lignification. In. *INRA-Reims Plant Chemistry Seminar Series*, INRA-Reims, Reims, France

Ralph, J. (2012) 'New' genes from novel plants for altering lignification. In., INRA-Versailles, Paris, France

Ralph, J. (2012) Efficient Biomass Conversion: Delineating the Best Lignin-Monomer-Substitutes. In. *GCEP Distinguished Lecturer Series*, DuPont, Wilmington, DE

Ralph, J. (2012) Efficient Biomass Conversion: Delineating the Best Lignin-Monomer-Substitutes. In. *GCEP Distinguished Lecturer Series*, ExxonMobil, Clinton, NJ

Ralph, J. (2012) Efficient Biomass Conversion: Delineating the Best Lignin-Monomer-Substitutes. In. *GCEP Distinguished Lecturer Series*, General Electric Company, Niskayuna, NY

Ralph, J. (2012) 'New' genes from novel plants for altering lignification. In. *2012 Gordon Conference on Plant Cell Walls*, GRC, Colby College, Waterville, ME

Ralph, J. (2012) State of the art on wood and grass lignin structure. In. *V Seminar on Pulp and Paper. Lignin Biorefinery: from biomass to product*, Federal University of Viçosa, Viçosa, MG Brazil

Grabber, J. H., Tobimatsu, Y., Elumalai, S., Pan, X., and Ralph, J. (2012) Biomimetic cell wall model studies to identify new lignin bioengineering targets for improving biomass susceptibility to pretreatment and enzymatic saccharification. In. *ASA, CSSA, and SSSA International Annual Meetings*, Cincinnati, OH.

Grabber, J. H., Tobimatsu, Y., Elumalai, S., Pan, X., and Ralph, J. (2012) Epigallocatechin gallate incorporation into lignin enhances the inherent fermentability, alkaline degradability, and enzymatic saccharifiability of artificially lignified maize cell walls. In. *ASA, CSSA, and SSSA International Annual Meetings*, Cincinnati, OH

Bonawitz, N. D., Li, X., Tobimatsu, Y., and Chapple, C. (2012) The transcriptional coregulatory complex Mediator is required for the dwarf phenotype of a lignin-deficient Arabidopsis mutant. In. *3rd Banff Conference on Plant Metabolism*, U. Calgary, The Banff Center, Banff, Alberta, Canada

Azarpira, A., Lu, F., Boerjan, W., Mansfield, S. D., Garlock, R., Dale, B. E., Ralph, J., Hedenström, M., and Tobimatsu, Y. (2012) Lignin (and extractable materials) from AFEX-treated wild type vs. transgenic poplars. In. *243rd International Meeting of American Chemical Society*, San Diego, CA

Y. Zhu, Y. Tobimatsu, S. Elumalai, H. Kim, C. Davidson, X. Pan, J. H. Grabber and J. Ralph. Potential monolignol replacements: Lignification with 4-hydroxycinnamoyl amides. 7th Annual Global Climate and Energy Project (GCEP) Research Symposium, Stanford, CA, 2011.

Y. Tobimatsu, S. Elumalai, J. H. Grabber, C. Davidson, X. Pan and J. Ralph. Hydroxycinnamate conjugates as potential monolignol replacements: in vitro lignification and cell wall studies with rosmarinic acid. 7th Annual Global Climate and Energy Project (GCEP) Research Symposium, Stanford, CA, 2011.

J. Ralph, F. Lu, Y. Tobimatsu, Y. Zhu, D. K. Ress, J. Rencoret, H. Kim, C. G. Wilkerson, S. Withers, S. D. Mansfield, J.-Y. Park, J. C. Sedbrook, R. A. Dixon, F. Chen, W. Boerjan, A. Wagner, C. Chapple, J.-K. Weng, J. H. Grabber, X. Pan, S. Elumalai, R. D. Hatfield and C. Davidson. Introducing novel linkages into lignin. Lignin: Characterization, Extraction, and Adding Value: EBI and JBEI Joint Workshop, JBEI, Emeryville, CA, 2011.

J. Ralph, F. Lu, H. Kim, Y. Zhu, J. Rencoret Pazo, J. H. Grabber, R. D. Hatfield, N. Santoro, C. Foster, R. Garlock, S. Chundawat, L. Sousa, V. Balan, B. E. Dale, W. Boerjan, J. C. Sedbrook, S. D. Mansfield, and C G. Wilkerson. Progress in Altering Plant Cell Wall Biosynthesis for Improved Biomass Processing. In. *JBEI post SAC Seminar*, JBEI, Emeryville, CA, 2011.

J. Ralph, F. Lu, H. Kim, Y. Zhu, J. Rencoret Pazo, Y. Tobimatsu, J. H. Grabber, R. D. Hatfield, J. C. Sedbrook, S. D. Mansfield, C. G. Wilkerson and W. Boerjan. Progress in Altering Plant Cell Wall Biosynthesis for Improved Biomass Processing. UBC Botany Seminar, UBC, Vancouver, Canada, 2011.

J. Ralph, F. Lu, H. Kim, Y. Zhu, J. Rencoret Pazo, Y. Tobimatsu, J. H. Grabber, R. D. Hatfield, J. C. Sedbrook, W. Boerjan, S. D. Mansfield and C. G. Wilkerson. Progress in Altering Plant Cell Wall Biosynthesis for Improved Biomass Processing. Novozymes Seminar, Novozymes, Raleigh, NC, 2011.

J. Ralph, F. Lu, H. Kim, Y. Zhu, J. Rencoret Pazo, J. H. Grabber, R. D. Hatfield, N. Santoro, C. Foster, R. Garlock, S. Chundawat, L. Sousa, V. Balan, B. E. Dale, W. Boerjan, J. C. Sedbrook, S. D. Mansfield and C. G. Wilkerson. Progress in Altering Plant Cell Wall Biosynthesis for Improved Biomass Processing. JBEI post SAC Seminar, JBEI, Emeryville, CA, 2011.

P. Mitra, F. Yang, J. S. Kim, J. Ito, H. Joshi, Y. Tobimatsu, A. Eudes, P. I. Benke, A. Mukhopadhyay, J. D. Keasling, J. Ralph, J. Heazelwood and L. Dominique. Biosynthesis of rosmarinic acid in Arabidopsis stems to engineer cleavable lignin. International Synthetic Biology Workshop: A Bio-based Future, Berkeley, CA, 2011.

S. Elumalai, J. H. Grabber, D. K. Ress, J. Ralph and X. Pan. Response to pretreatment and enzymatic digestibility of cell walls lignified with normal and novel lignin precursors. 16th International Symposium on Wood, Fiber, and Pulping Chemistry (ISFWPC), Tanjing, China, 2011.

S. Elumalai, J. H. Grabber, J. Ralph and X. Pan. Evaluating the response of artificially lignified maize cell wall to pretreatments and enzymatic hydrolysis. 16th International Symposium on Wood, Fiber, and Pulping Chemistry, Tanjing, China, 2011.

Y. Zhu, Y. Tobimatsu, J. H. Grabber and J. Ralph. Potential monolignol replacements: Synthesis and lignification of β-hydroxycinnamoyl glucosides and monolignol γ-glucosides. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.

Y. Tobimatsu, Y. Zhu, H. Kim, F. Lu, J. H. Grabber and J. Ralph. Potential monolignol replacements: Lignification with guaiacyl butenol, feruloyl methoxytyramine, and rosmarinic acid. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.

D. K. Ress, J. N. Rehbeck, W. Maier, H. Kim, F. Lu, J. Ralph, C. Davidson and J. H. Grabber. Potential monolignol replacements: Synthetic strategies toward phenolic and diferulate esters of flavan-3-ols, tartaric acid and glucose. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.

J. Ralph, X. Pan, S. Patterson, F. Lu, H. Kim, Y. Zhu, D. K. Ress, M. Opietnik, Y. Tobimatsu, T. Fabel, S. Elumali, C. Davidson and J. H. Grabber. Altering lignin biosynthesis for improved biomass conversion [Efficient biomass conversion: Delineating the best lignin monomer-substitutes]. Global Climate and Energy Project (GCEP) Working Group Meeting, Stanford, CA, 2010.

J. Ralph, H. Kim, F. Lu, D. K. Ress, Y. Tobimatsu, X. Pan, S. Patterson and J. H. Grabber. Efficient biomass conversion: delineating the best monolignol substitutes (introductory overview). Global Climate & Energy Project, Stanford University, 2010.
M. Opietnik, P. Schatz, H. Kim, C. Davidson, J. H. Grabber and J. Ralph. Potential monolignol replacements: Synthesis of feruloyl-quinic acids and feruloyl-threitols. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.
J. H. Grabber, J. Ralph, D. K. Ress and X. Pan. Incorporation of epicatechin esters into lignin enhances cell wall fermentability. 32nd Symposium on Biotechnology for Fuels and Chemicals, Hilton Clearwater Beach, Clearwater, FL, 2010, Paper #15139.
J. H. Grabber, J. Ralph and X. Pan. Identifying new lignin bioengineering targets: Monolignol substitute impacts on lignin formation and cell wall utilization. 32nd Symposium on Biotechnology for Fuels and Chemicals, Hilton Clearwater Beach, Clearwater, FL, 2010, Paper #15069.
S. Elumalai, J. H. Grabber, J. Ralph and X. Pan. Microscale analysis and pretreatment methods for evaluating the delignification response of artificially lignified cell walls containing novel monolignols. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.
J. Bolivar, T. Fabel, S. Patterson, J. H. Grabber and J. Ralph. In vitro culture systems to test lignification and cell wall digestibility. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.
D. K. Ress, Y. Tobimatsu, H. Kim, F. Lu, J. Ralph, C. Davidson and J. H. Grabber. Development of a Systems Based Lignification Archetype Using Unconventional Lignin Monomer Substitutes. Global Climate & Energy Project, Stanford University, 2009.
J. Ralph, H. Kim, F. Lu, D. K. Ress, Y. Tobimatsu, X. Pan, S. Patterson and J. H. Grabber. Efficient biomass conversion: delineating the best monolignol substitutes (Introductory overview). Global Climate & Energy Project, Stanford University, 2009.
J. Ralph, J. H. Grabber, F. Lu, H. Kim, J. M. Marita and R. D. Hatfield. Incorporation of monolignol conjugates into lignin for improved processing. 2009 IUFRO Tree Biotechnology Conference, Whistler, BC, Canada, 2009, 11.
J. Ralph. The future for liquid biofuels from woody biomass and other cellulosic matter. EECA Biofuels and Electric Vehicles Conference 2009, Te Papa, Wellington, New Zealand, 2009.
J. Ralph. Modification of cell wall structure to improve biomass processing. Scion Seminar Series, Scion, Rotorua, New Zealand, 2009.
J. Ralph, R. D. Hatfield, J. H. Grabber, F. Lu, H. Kim and J. M. Marita. Designing lignins for improved biomass processing. FuncFiber 2008 International Symposium on the biology and biotechnology of wood, Umeå, Sweden, 2008.
J. Ralph, J. H. Grabber, F. Lu, H. Kim, J. M. Marita and R. D. Hatfield. Designing lignins for improved biomass processing. Global Climate and Energy Change (GCEP) Research Symposium, Frances C. Arrillaga Alumni Center, Stanford University, Stanford, CA, 2008.
J. Ralph. A basis for... Overcoming the 'lignin problem' in biomass conversion. 2008 Contemporary Biochemistry, Biofuels Seminar Series, Ebling Symposium Center, Microbial Sciences Building, U. Wisconsin-Madison, 2008.

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References

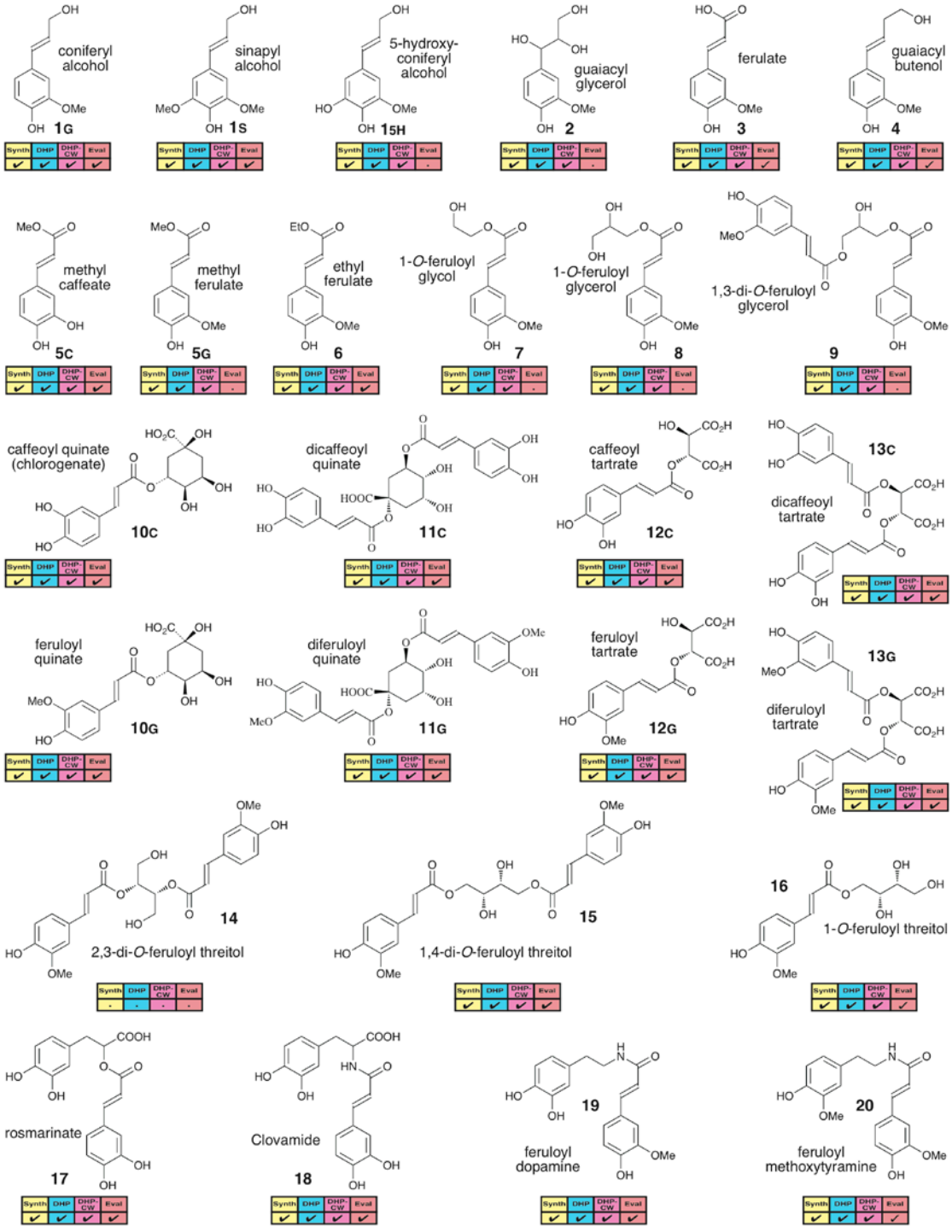
1. Y. Tobimatsu, S. Elumalai, J. H. Grabber, C. L. Davidson, X. Pan and J. Ralph. Hydroxycinnamate conjugates as potential monolignol replacements: *in vitro* lignification and cell wall studies with rosmarinic acid. *ChemSusChem*, 2012, **5**, 676-686 and COVER ARTICLE.
2. J. H. Grabber, D. K. Ress and J. Ralph. Identifying new lignin bioengineering targets: Impact of epicatechin, quercetin glycoside, and gallate derivatives on the lignification and fermentation of maize cell walls. *Journal of Agricultural and Food Chemistry*, 2012, **60**, 5152-5160.
3. J. H. Grabber, P. F. Schatz, H. Kim, F. Lu and J. Ralph. Identifying new lignin bioengineering targets: 1. Monolignol substitute impacts on lignin formation and cell wall fermentability. *BMC Plant Biology*, 2010, **10**, 1-13.
4. S. Elumalai, Y. Tobimatsu, J. H. Grabber, X. Pan and J. Ralph. Epigallocatechin gallate incorporation into lignin enhances the alkaline delignification and enzymatic saccharification of cell walls. *Biotechnology for Biofuels*, 2012, **5**, 59.
5. R. Vanholme, J. Ralph, T. Akiyama, F. Lu, J. Rencoret Pazo, J. Christensen, A. Rohde, K. Morreel, R. DeRycke, H. Kim, B. Van Reusel and W. Boerjan. Engineering traditional monolignols out of lignins by concomitant up-regulation *F5H1* and down-regulation of *COMT* in Arabidopsis *The Plant Journal*, 2010, **64**, 885-897 and COVER ARTICLE.
6. Y. Zhu, A. Mohammadi and J. Ralph. Facile synthesis of 4-hydroxycinnamaldehydes. *BioEnergy Research*, 2012, **5**, 407-411.
7. D. K. Ress and J. Ralph. Synthesis of 3-O-vanillate and 3-O-ferulate esters of (-)-epicatechin. *Journal of Agricultural and Food Chemistry*, 2012, submitted 8/25/2011; needs updating with DHPs.
8. Y. Zhu and J. Ralph. Stereoselective synthesis of 1-*O*-β-feruloyl and 1-*O*-β-sinapoyl glucopyranoses. *Tetrahedron Letters*, 2011, **52**, 3729-3731.
9. Y. Tobimatsu, C. L. Davidson, J. H. Grabber and J. Ralph. Fluorescence-tagged monolignols: Synthesis and application to studying *in vitro* lignification. *Biomacromolecules*, 2011, **12**, 1752-1761.
10. Y. Tobimatsu, A. Wagner, L. Donaldson, P. Mitra, D. Loque, C. Niculaes, O. Dima, J.-I. Kim, N. A. Anderson, C. Chapple, W. Boerjan and J. Ralph. Probing plant cell wall lignification with fluorescence-tagged monolignols. *The Plant Journal*, 2013, submitted (May 2013).
11. K. Morreel, O. Dima, H. Kim, F. Lu, C. Niculaes, R. Vanholme, R. Dauwe, G. Goeminne, D. Inzé, E. Messens, J. Ralph and W. Boerjan. Mass-spectrometry-based sequencing of lignin oligomers. *Plant Physiology*, 2010, **153**, 1464-1478.
12. K. Morreel, H. Kim, F. Lu, O. Dima, T. Akiyama, R. Vanholme, C. Niculaes, G. Goeminne, D. Inzé, E. Messens, J. Ralph and W. Boerjan. Mass-spectrometry-based fragmentation as an identification tool in lignomics. *Analytical Chemistry*, 2010, **82**, 8095-8105.
13. K. Morreel, J. Ralph, F. Lu, G. Goeminne, R. Busson, P. Herdewijn, J. L. Goeman, J. Van der Eycken, W. Boerjan and E. Messens. Phenolic profiling of caffeic acid *O*-methyltransferase-deficient poplar reveals novel benzodioxane oligolignols. *Plant Physiology*, 2004, **136**, 4023-4036.
14. K. Morreel, J. Ralph, H. Kim, F. Lu, G. Goeminne, S. A. Ralph, E. Messens and W. Boerjan. Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem. *Plant Physiology*, 2004, **136**, 3537-3549.
15. R. Vanholme, K. Morreel, C. Darrah, P. Oyarce, J. H. Grabber, J. Ralph and W. Boerjan. Metabolic engineering of novel lignin in biomass crops. *New Phytologist*, 2012, **196**, 978-1000.
16. J. Ralph. Perturbing Lignification. in *The Compromised Wood Workshop 2007*, eds. K. Entwistle, P. J. Harris and J. Walker, Wood Technology Research Centre, University of Canterbury, New Zealand, Canterbury, Editon edn., 2007, pp. 85-112.
17. J. Ralph. What makes a good monolignol substitute? in *The Science and Lore of the Plant Cell Wall Biosynthesis, Structure and Function*, ed. T. Hayashi, Universal Publishers (BrownWalker Press), Boca Raton, FL, Editon edn., 2006, pp. 285-293.
18. J. Ralph, Lundquist, K., Brunow, G. Lu, F., Kim, H., Schatz, P. F., Marita, J. M., Hatfield, R. D., Ralph, S. A. Christensen, J. H., Boerjan, W. Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochemistry Reviews*, 2004, **3**, 29-60.
19. W. Boerjan, J. Ralph and M. Baucher. Lignin biosynthesis. *Annual Reviews in Plant Biology*, 2003, **54**, 519-549.
20. R. Vanholme, K. Morreel, J. Ralph and W. Boerjan. Lignin engineering. *Current Opinion in Plant Biology*, 2008, **11**, 278-285.

21. J. Ralph, C. Lapierre, J. Marita, H. Kim, F. Lu, R. D. Hatfield, S. A. Ralph, C. Chapple, R. Franke, M. R. Hemm, J. Van Doorsselaere, R. R. Sederoff, D. M. O'Malley, J. T. Scott, J. J. MacKay, N. Yahiaoui, A.-M. Boudet, M. Pean, G. Pilate, L. Jouanin and W. Boerjan. Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry*, 2001, **57**, 993-1003.
22. J. Ralph, H. Kim, F. Lu, J. H. Grabber, J.-C. Leplé, J. Berrio-Sierra, M. Mir Derikvand, L. Jouanin, W. Boerjan and C. Lapierre. Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl-CoA reductase deficiency). *The Plant Journal*, 2008, **53**, 368-379.
23. F. Chen, Y. Tobimatsu, D. Havkin-Frenkel, R. A. Dixon and J. Ralph. A polymer of caffeyl alcohol in plant seeds. *Proceedings of the National Academy of Sciences of the United States of America*, 2012, **109**, 1772-1777 and COVER ARTICLE; Editors' choice highlight in *Science*, 1**335**, 1382 (2012).
24. S. Withers, F. Lu, H. Kim, Y. Zhu, J. Ralph and C. G. Wilkerson. Identification of a grass-specific enzyme that acylates monolignols with *p*-coumarate. *Journal of Biological Chemistry*, 2012, **287**, 8347-8355.
25. S. Withers, Y. Zhu, F. Lu, H. Kim, J.-Y. Park, S. D. Mansfield, C. G. Wilkerson and J. Ralph. Identification of enzymes that produce acylated monolignols: Progress in the pursuit of zip-lignin. 2012 DOE Genomic Science Meeting, Bethesda, MD, 2012.
26. S. Withers, F. Lu, J. Ralph and C. G. Wilkerson. Cloning and characterization of an acyltransferase that synthesizes monolignol ferulate conjugates for generation of 'zipped' lignins. 2011 Genomic Science Awardee Meeting IX (GTL) meeting and USDA-DOE Plant Feedstock Genomics for Bioenergy Awardee Meeting, Crystal City, Virginia, 2011.
27. J. Ralph, C. G. Wilkerson, F. Lu, H. Kim, Y. Zhu, J. Rencoret, J. H. Grabber, R. D. Hatfield, J. C. Sedbrook, W. Boerjan and S. D. Mansfield. Approaches toward improving bioenergy crops for efficient biomass conversion. 7th International Conference on Renewable Resources and Biorefineries, Brugges, Belgium, 2011.
28. J. Ralph, F. Lu, H. Kim, Y. Zhu, J. Rencoret Pazo, J. H. Grabber, R. D. Hatfield, N. Santoro, C. Foster, R. Garlock, S. Chundawat, L. Sousa, V. Balan, B. E. Dale, W. Boerjan, J. C. Sedbrook, S. D. Mansfield and C. G. Wilkerson. Altering lignin biosynthesis for improved biomass processing. 26th New Phytologist Symposium: Bioenergy trees, INRA-Nancy, France, 2011, paper 3.6.
29. J. Ralph, J. H. Grabber, R. D. Hatfield, F. Lu, H. Kim, J. M. Marita and C. G. Wilkerson. Incorporation of monolignol conjugates into lignin for improved processing. XII Cell Wall Meeting, Porto, Portugal, 2010, Paper T12, p. 18.
30. I. A. Larskaya, F. Lu, J. Ralph, C. G. Wilkerson and S. Withers. Cloning and characterization of an acyltransferase that synthesizes coniferyl ferulate. XII Cell Wall Meeting, Porto, Portugal, 2010, Paper P89, p. 108.
31. J. Ralph. Hydroxycinnamates in Lignification. *Phytochemistry Reviews*, 2010, **9**, 65-83.
32. J. H. Grabber, R. D. Hatfield, F. Lu and J. Ralph. Coniferyl ferulate incorporation into lignin enhances the alkaline delignification and enzymatic degradation of maize cell walls. *Biomacromolecules*, 2008, **9**, 2510-2516.
33. J.-K. Weng, T. Akiyama, J. Ralph, B. L. Golden and C. Chapple. Independent recruitment of an *O*-methyltransferase for syringyl lignin biosynthesis in *Selaginella moellendorffii*. *Plant Cell*, 2011, **23**, 2708-2724.
34. J.-K. Weng, T. Akiyama, N. D. Bonawitz, X. Li, J. Ralph and C. Chapple. Convergent evolution of syringyl lignin biosynthesis via distinct pathways in the lycophyte *Selaginella* and flowering plants. *Plant Cell*, 2010, **22**, 1033-1045.
35. J.-K. Weng, X. Li, J. Stout and C. Chapple. Independent origins of syringyl lignin in vascular plants. *Proceedings of the National Academy of Sciences*, 2008, **105**, 7887-7892.
36. M. Petersen and M. S. J. Simmonds. Molecules of interest - Rosmarinic acid. *Phytochemistry*, 2003, **62**, 121-125.
37. B. E. Ellis and G. H. N. Towers. Biogenesis of rosmarinic acid in *Mentha*. *Biochemical Journal*, 1970, **118**, 291-297.
38. C. Landmann, S. Huecherig, B. Fink, T. Hoffmann, D. Dittlein, H. A. Coiner and W. Schwab. Substrate promiscuity of a rosmarinic acid synthase from lavender (Lavandula angustifolia L.). *Planta*, 2011, **234**, 305-320.

39. M. Sander and M. Petersen. Distinct substrate specificities and unusual substrate flexibilities of two hydroxycinnamoyltransferases, rosmarinic acid synthase and hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl-transferase, from Coleus blumei Benth. *Planta*, 2011, **233**, 1157-1171.
40. C. Weitzel and M. Petersen. Cloning and characterisation of rosmarinic acid synthase from Melissa officinalis L. *Phytochemistry*, 2011, **72**, 572-578.
41. J. H. Grabber, J. Ralph, D. K. Ress and X. Pan. Incorporation of epicatechin esters into lignin enhances cell wall fermentability. 32nd Symposium on Biotechnology for Fuels and Chemicals, Hilton Clearwater Beach, Clearwater, FL, 2010, Paper #15139.

(Next two pages)

Figure 1. Compounds, synthesized or acquired, for lignification studies. Names are on all of the compounds. The color coded section under each compound is to indicated whether: ***Synth*** – it has been obtained (synthesized or acquired); ***DHP*** – it has been subjected to *in vitro* lignification, coupled with NMR to determine whether it is compatible with lignification (and cross-couples into the polymer); ***DHP-DW*** – it has been incorporated (along with the traditional monolignols, coniferyl and sinapyl alcohols **1** into lignins within the suspension-cultured corn cell wall system, as well a subjected to NMR to again characterize the *in muro* cross-coupling; and ***Eval*** – the cell walls have undergone (or are undergoing) analysis, pretreatment, and enzymatic saccharification to determine the efficacy of the monomer substitution. A lighter tick indicates partial progress.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sinapyl alcohol
1S
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1SH
guaiacyl glycerol
2
ferulate
3
guaiacyl butenol
4
methyl caffeate
5C
methyl ferulate
5G
ethyl ferulate
6
1-O-feruloyl glycol
7
1-O-feruloyl glycerol
8
1,3-di-O-feruloyl glycerol
9
caffeoyl quinate (chlorogenate)
10C
dicaffeoyl quinate
11C
caffeoyl tartrate
12C
13C
dicaffeoyl tartrate
feruloyl quinate
10G
diferuloyl quinate
11G
feruloyl tartrate
12G
13G
diferuloyl tartrate
14
2,3-di-O-feruloyl threitol
15
1,4-di-O-feruloyl threitol
16
1-O-feruloyl threitol
rosmarinate
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Synth
DHP
DHP-CW
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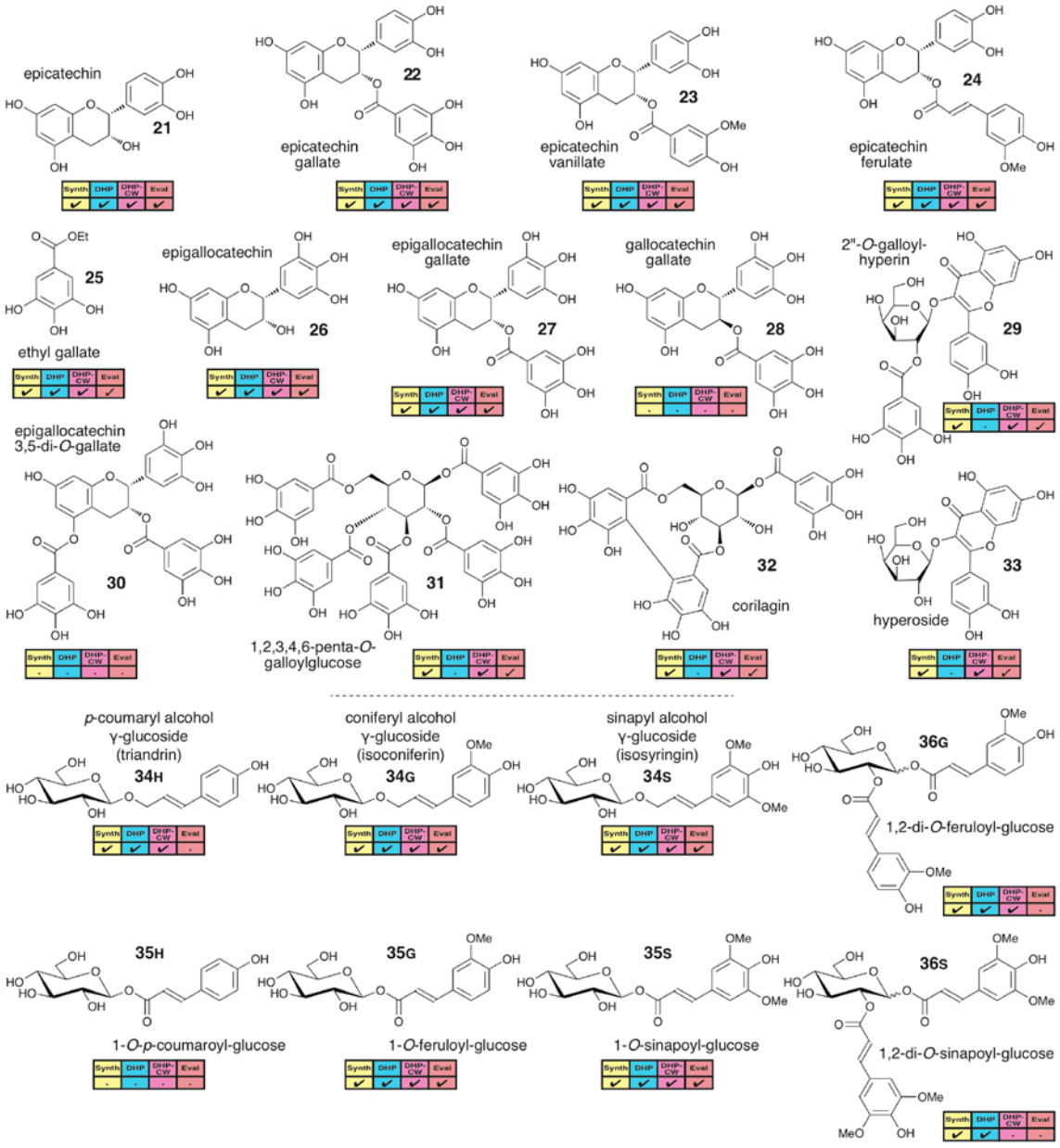
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21
22
epicatechin gallate
23
epicatechin vanillate
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25
ethyl gallate
epigallocatechin
26
epigallocatechin gallate
27
gallocatechin gallate
28
2"-O-galloyl-hyperin
29
epigallocatechin 3,5-di-O-gallate
30
31
1,2,3,4,6-penta-O-galloylglucose
32
corilagin
33
hyperoside
p-coumaryl alcohol γ-glucoside (triandrin)
34H
coniferyl alcohol γ-glucoside (isoconiferin)
34G
sinapyl alcohol γ-glucoside (isosyringin)
34S
36G
1,2-di-O-feruloyl-glucose
35H
1-O-p-coumaroyl-glucose
35G
1-O-feruloyl-glucose
35S
1-O-sinapoyl-glucose
36S
1,2-di-O-sinapoyl-glucose
Synth
DHP
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Eval