Lignin Management:
Optimizing Yield in Lignin-Modified Plants

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Abstract

This project aims to maximize the utility of plant lignocellulosic biomass as an abundant, sustainable, and carbon-neutral energy feedstock by optimizing both its yield and composition to facilitate downstream conversions to fuel and electricity. Working independently with different lignin-deficient mutants, the partners have discovered novel genes that mitigate the growth defects [so-called lignin-modification-induced dwarfism (LMID)] seen in severely lignin-depleted plants. Revealing the mechanism(s) by which this mitigation occurs is critical to fundamental understanding and useful manipulation of how plants partition carbon and may enable biomass manipulation for carbon sequestration in the future. We have undertaken several projects to determine the causes of, and to reduce the effects of, LMID. By expressing the lignin biosynthetic gene *CSE* only in vessel elements, the effects of LMID in *cse* mutants is lessened. We are also utilizing mutant screens in lignin biosynthetic mutants to discover novel genes involved in LMID. One screen, in the *ccr1* background, has led to the discovery of a mutant that partially restores the growth defect of the original line, yet maintains saccharification efficiency. The gene responsible for this trait has been identified, and tests are underway to understand the mechanism behind the LMID reduction. Another mutant screen, in the highly dwarfed *ref8* (*c3h*) background, has identified more than 20 lines that suppress LMID, designated as *growth inhibition relieved (gir)*. One of these, *GIR1*, has been identified through a mapping by next generation

sequencing approach as a β-importin important for translocation of a transcription factor responsible for regulation of lignin biosynthesis genes. Another screen for suppressors of a mutant in the transcriptional complex Mediator, which controls the LMID response in *ref8* plants, has yielded a variety of mutants which could lead to a greater understanding of how LMID is induced in *c3h* plants.

Towards identifying the pathways responsible for LMID, a metabolomic pipeline has been established to identify metabolites altered in lignin mutants. The detection and authentication of compounds identified by this pipeline will be enhanced because we have synthesized several lignin trimers and tetramers, allowing us to generate metabolite profiles for them. Several phenylpropanoid metabolites were screened for their ability to affect *Arabidopsis* growth, and one, *cis*-cinnamic acid, was found to alter growth, most likely through auxin pathway perturbation. The incorporation of alternative monolignols has also been investigated, as the inclusion of hydroxycinnamaldehydes leads to an increase in saccharification potential. We are also targeting the orthologs of high saccharification mutants previously identified in *Arabidopsis* for implementation in energy crops (barley, poplar). The new CRISPR/Cas9 technology will allow for targeted knock-outs of lignin biosynthesis genes in barley. This will allow for greater effects on plant lignin content and composition.

Introduction

The Sun is the principal source of energy for our planet, and plant biomass is the major vector by which that energy is captured and stored for human exploitation. This project aims to maximize the utility of plant lignocellulosic biomass as an abundant, sustainable, and carbon-neutral energy feedstock by optimizing both its yield and composition to facilitate downstream conversions to fuel and electricity. Working independently with different lignin-deficient mutants, the partners have discovered novel genes that mitigate the LMID seen in severely lignin-depleted plants. Revealing the mechanism(s) by which this mitigation occurs is critical to fundamental understanding and useful manipulation of how plants partition carbon and may enable biomass manipulation for carbon sequestration in the future. Up to now, much of this work has been performed in the model plant Arabidopsis. However several partners (WB, CC, CH) have developed the capability to make similar genetic changes in poplar and barley, two genuine crop plants with world-wide distribution that are also recognized as ideal models for other trees and grasses, respectively. This project is intended to enable maximal lignin modifications to be generated in plants where normal vascular integrity, strength and disease-resistance are maintained. Deploying these game-changing modifications coupled with the new discoveries arising from this project in crop plants (initially barley) will open the door for scalability in other agricultural and energy crops. With large-scale deployment and the improved economics enabled by lignin optimization, these advances will underpin the development of cost-efficient biomass-based biofuels and achieve substantial reductions in global greenhouse gas emissions.

Background

In recent years, many studies have highlighted the potential for improving biofuel production. As the phenolic polymer lignin coats and is cross-linked to other cell wall

components, the reduction or alteration of this compound has long been a target for improving sugar release from cell walls. Recently, new targets for the reduction of lignin have been identified. A screen for improved saccharification has generated several mutants with alterations in cell wall composition in *Brachypodium distachyon.*[1] A transcriptional repressor has been shown to affect expression of lignin genes, including *C4H* and *CCR1*, and, consequently lignin content.[2] The importance of monolignol biosynthesis has been shown in poplar also, as reductions in cinnamoyl-CoA reductase (CCR) in poplar increase saccharification efficiency.[3] These studies highlight the range of targets that can be manipulated to improve saccharification properties. However, disruptions in lignin genes (and others) can lead to reduced growth.

Understanding the molecular mechanism(s) whereby suppressor mutations can restore the biomass yield of reduced-lignin CCR-, C4H- or C3H-deficient plants is of crucial and fundamental importance as it will reveal the basic principles whereby plants partition carbon to lignin and other metabolic compartments. Knowledge of these mechanisms will open up valuable opportunities for greater useful manipulations to lignin either by maximizing yield in saccharification-optimized plants, or increasing carbon allocation and storage capacity in organs such as roots to sequester carbon in soils for longer durations. Fundamental and novel research to illuminate these mechanisms is a major feature of the current work. Although it is possible that LMID may come about via more than one mechanism, an understanding of one of them has been achieved by partner Chapple via his recent discovery that the Mediator complex influences carbon allocation to the lignin and phenylpropanoid biosynthetic pathways by transcriptional co-activation or co-suppression of suites of co-regulated genes. Powerful tools, including revolutionary techniques for speeding up the identification of unknown metabolites are being used to further investigate these mechanisms.

Results

1. Determine the mechanisms and genes required for LMID

The *CSE* lignin biosynthetic gene recently discovered in our laboratories is a potential candidate for biotechnology use in crops, in order to minimize pretreatment cost in the biorefinery.[4] We have shown that the Arabidopsis knockout mutant *cse-2* deposits 36% less lignin and, upon saccharification without pretreatment, releases 75% more glucose per plant than the wild type. On the other hand, *cse-2* mutants were 37% smaller and 42% lighter than the wild type.[4] Potentially, the observed LMID in *cse-2* mutants could be attributed to xylem vessel collapse, which results in less efficient transport of water and nutrients. To test this hypothesis, we expressed *CSE* under a vessel-specific promoter in a *cse-2* background, to allow lignification in the vessel, but not in the fiber cells, similar to a previous study on *c4h.*[5] We used the vessel specific promoters of the *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7* transcription factor genes. *VND6* and *VND7* encode NAM/ATAF/CUC (NAC) domain protein transcription factors and act as key regulators of metaxylem and protoxylem vessel differentiation, respectively.[5,6]

The *cse-2* mutant was transformed with *proVND6::CSE* and *proVND7::CSE* (known as *VND6* and *VND7* in this document). Analysis of the growth phenotype indicate that *VND7* lines perform better than *VND6* lines. As anticipated, the final dry weights of the main stem

of all four *cse-2 proVND7::CSE* lines were significantly higher (up to 30%) than that of the *cse-2* mutant, but were not significantly different from that of the wild type. However, the final height was only partially restored in these lines, with inflorescence stems still being between ~10% shorter than those of the wild type. Nevertheless, these lines yielded plants that were still significantly taller (up to 51%) than *cse-2* plants, whose inflorescence stems were 38% shorter than those of the wild type. Similar results were obtained for the *cse-2 proVND6::CSE* lines, with fully complemented weight but partially restored height. Thus, the recovery of the yield penalty of the *cse-2* mutant is substantial but not complete, by specifically targeting *CSE* expression to the vessels.

Total acetyl bromide (AcBr) lignin amounts for all *cse-2 proVND7::CSE* lines were significantly lower than that of the wild type. H:G:S ratios, as estimated from thioacidolysis, were about 1:62:37 in the wild type, and about 50:25:25 in the *cse-2* mutant, reflecting the large increase in H units in the *cse-2* mutant. The H:G:S ratio of the *cse-2 proVND7::CSE* lines did not differ significantly from those of *cse-2.* Subsequently, senesced stems of the wild-type, *cse-2* mutant, and the *cse-2 proVND7::CSE* lines were saccharified without pretreatment. The data revealed that the cellulose-to-glucose conversion was significantly higher for all *cse-2 proVND7::CSE* lines than for wild type. As anticipated, Glc release of the vessel-specific complemented lines were higher (up to 36%) when compared to that of the non-complemented *cse-2* mutant and up to 150% higher when compared to that of the wild type.

In a parallel approach, a screen for LMID rescue was performed on the C3H-defective mutant, *ref8*. Because *ref8* exhibits severe LMID and is sterile, a *ref8* mutant line (*ref8*^{pOpON}) carrying a pOpON C3H construct which enables chemically inducible expression of C3H was generated.[7] When sprayed with dexamethasone, *ref8*^{pOpON} grew significantly better and was fertile, whereas in the absence of the chemical inducer, *ref8*^{pOpON} behaved the same as the original *ref8* mutant. The bulked seeds for *ref8*^{pOpON} were mutagenized and grown with dexamethasone induction to obtain M2 seeds. M2 plants that had the ability to grow relatively normally in the absence of dexamethasone were identified as *ref8* suppressors.

Screening of M2 plants identified more than 20 suppressor lines, and we have been working to map the suppressor genes, named as *growth inhibition relieved (gir)*, by next-generation-sequencing-based bulked segregant analysis.[8] We backcrossed a recessive suppressor to the original *ref8*^{pOpON} line to generate a F2 mapping population. The F2 plants segregated to two phenotypic groups, *ref8* and suppressor. For each group, we pooled at least fifty individual plants and extracted DNA from the bulked plants. The two DNA samples were subjected to Illumina sequencing and mutations were identified for each of the two DNA samples by comparing the sequence data with the Arabidopsis Col-0 genome. Candidate genes were identified based on the expected allele frequency of the causal mutation in the two bulked samples. So far, we have generated the F2 mapping population for sixteen suppressor lines and have finished sequencing for twelve. Five of these twelve suppressor lines mapped to the same gene, representing multiple alleles of the *GIR1* gene. We have performed a complementation test, and we have generated a double mutant from

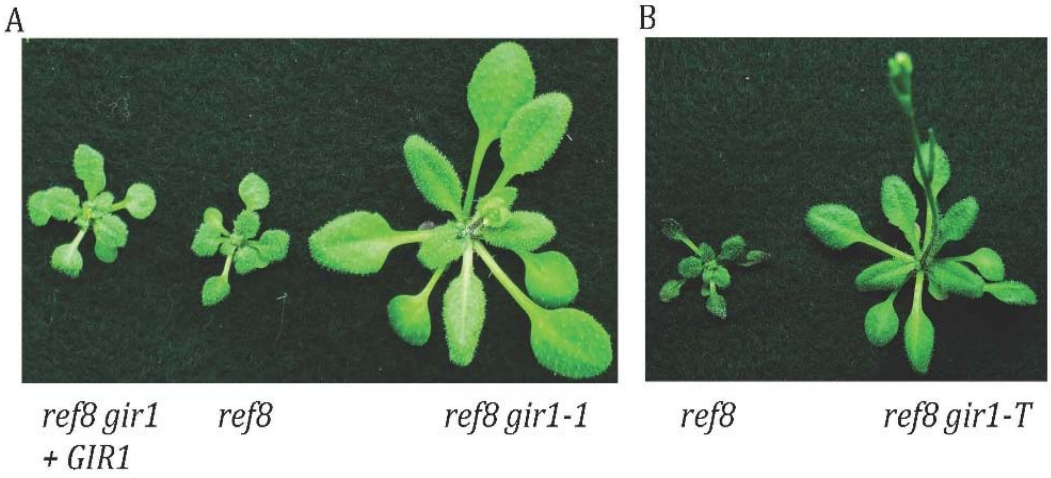

Figure 1: Evidence that *gir1* is a suppressor of *ref8* as demonstrated by functional complementation (A) and generation of a double mutant using a t-DNA allele (B).

ref8 and a T-DNA mutant of *GIR1* (*gir1-T*). These experiments verified that loss of function of *GIR1* is responsible for the growth phenotype of the suppressor (Figure 1).

GIR1 has been previously reported to encode an importin β–protein that mediates the transport of MYB4, a transcription factor that suppresses the phenylpropanoid pathway, from the cytosol into the nucleus.[9] Therefore, it is conceivable that loss of function of GIR1 would result in a nuclear import defect of MYB4 and thereby upregulates the phenylpropanoid pathway, countering the effect of *ref8*. To test this hypothesis, we measured the expression levels of *C4H*, a major gene suppressed by MYB4, as well as that of *C3H*, in wild type, *ref8*, *gir1*, and *ref8 gir1* plants. Surprisingly, no significant difference was observed in either *C4H* or *C3H* expression levels among these plants (Figure 2). The *ref8 gir1* plant has higher lignin content compared to *ref8* (Figure 3A). This is mainly due to the increases in H lignin (Figure 3B). These results suggest that *gir1* redistributed flux into H lignin rather than up-regulated the phenylpropanoid pathway. To unequivocally test if the

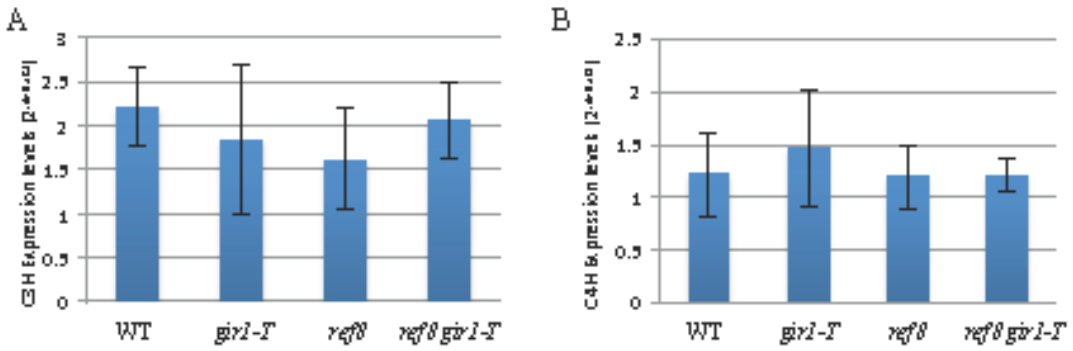

Figure 2: Gene expression levels determined by qRT-PCR for *C3H* (A) and *C4H* (B).

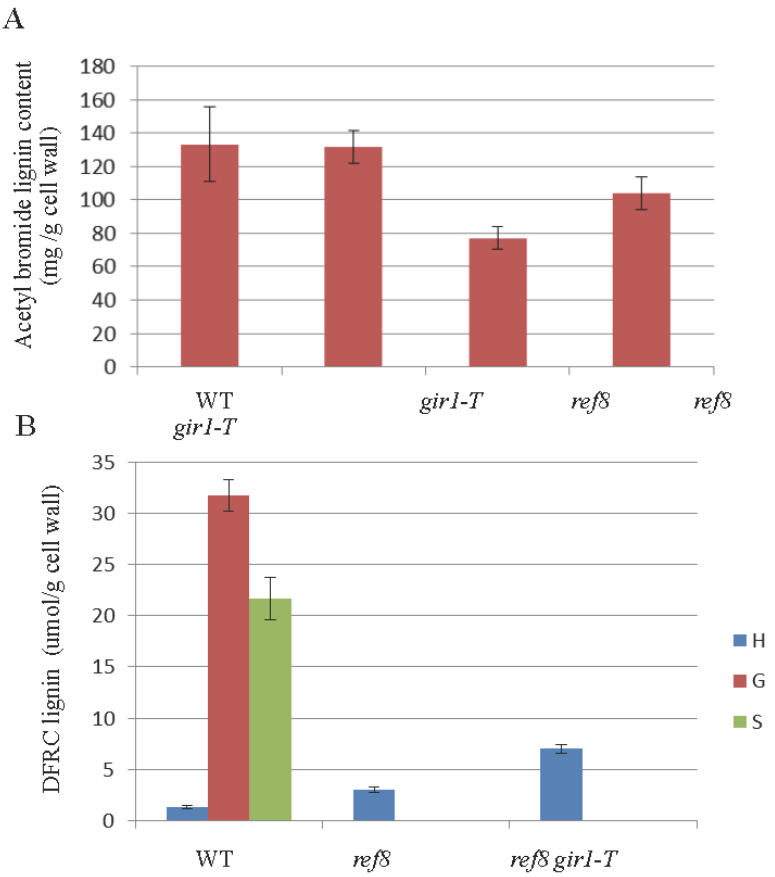

Figure 3: Lignin content (A) and composition (B) of wild type (WT), *ref8*, and *ref8 gir1-T*.

growth restoration effect of *gir1* is mediated through MYB4, we generated a loss-of-function *myb4* mutant using the CRISPR/Cas9 technology. We crossed the *myb4* mutant with *ref8* to generate the *myb4 ref8* double mutant. If MYB4 transportation were involved in growth restoration, the *myb4* mutant would rescue the dwarf phenotype of *ref8*. We have obtained F2 seeds from the cross. We expect to obtain a definite conclusion on the MYB4 hypothesis in a few months. We have also crossed *gir1-T* to Arabidopsis *ccr1* and *c4h* mutants to test whether GIR1 is involved in LMID of other lignin mutants. We have obtained F2 seeds and are in the process of identifying double mutants.

In addition to *GIR1*, we have also identified another two putative suppressor genes, *GIR2* and *GIR3*, which encode transcription factors. These new discoveries generate a new hypothesis that these transcription factors may require GIR1 to be transported into the nucleus. We have generated yeast two-hybrid constructs to test whether GIR2 or GIR3 physically interact with GIR1.

LMID greatly limits the deployment of current lignin engineering strategies for biofuel feedstock improvement. Although its mechanism remains unknown, it is now clear that

LMID is a genetically controlled process.[7,10] We have isolated more than twenty suppressor mutants of an Arabidopsis lignin mutant and made good progress in identifying *GIR* genes involved in LMID. It is expected that more *GIR* genes will be identified as the project continues. Further characterization of these *GIR* genes in the near future will provide us more clues to the underlying LMID mechanisms, enabling the development of novel strategies to optimizing yield in lignin-modified plants.

2. Identify the signaling and transcriptional changes that lead to LMID in c3h plants

We have previously shown that the REF4 and RFR1 subunits of the Mediator complex are required for LMID in *c3h* plants. Disruption of these genes does not restore growth to a C4H-deficient mutant, and tests with other dwarf lignin mutants are underway. Using RNAseq we showed that more than 30% of the genome is differentially expressed in *c3h* plants compared to wild-type plants. Interestingly, disruption of REF4 and RFR1 in the *c3h* mutant restored the transcription of almost 90% of these genes back to wild-type levels.[10] This has led us to propose a model whereby mutation of *c3h* produces a feedback signal, the interpretation of which requires REF4/RFR1 and that results in widespread transcriptional reprogramming and ultimately dwarfing. We are now working on understanding how these transcriptional changes lead to LMID. The extreme growth defects of *c3h* mutant plants and the resulting pleiotropic effects of these defects on normal gene expression confound the identification of immediate target genes that lead to LMID. To dissect the direct REF4/RFR1-dependent targets of Mediator responsible for dwarfing in *c3h* plants from downstream compensatory gene changes, we are using dexamethasone-inducible transgene constructs for REF4 and RFR1 (pOpOn REF4/RFR1) that will enable a time-resolved understanding of the transcriptional cascade that leads to LMID in the context of the *c3h* mutation.

We have continued work on this objective but have found that the T-DNA insertions in *med5a* and *med5b* mutants lead to silencing of the genes encoded within the pOpOn REF4/RFR1 T-DNA. As a result, we are now generating *med5a med5b* double mutants using point mutant nulls identified in various suppressor screens and will then transform them with pOpOn REF4/RFR1 constructs to complete this objective.

3. Reveal the direct targets of Mediator responsible for dwarfing in ref4-3 plants

In a screen for plants defective in phenylpropanoid biosynthesis, we identified a semi-dominant mutant of a subunit of the Mediator complex (*reduced epidermal fluorescence 4-3, ref4-3*) that accumulates low levels of phenylpropanoids and has a dwarf phenotype.[11] Plants that are null for both *REF4 (MED5a)* and its paralog *RFR1 (MED5b)* exhibit normal growth but accumulate increased levels of phenylpropanoids.[12,13] Later gene expression analysis showed that transcription of phenylpropanoid biosynthetic genes was correspondingly altered in the mutants.[10,13] More recently we have shown that *MED5* is required for transcriptional reprogramming in the low-lignin *ref8* mutant, and for metabolic cross-talk between the glucosinolate and phenylpropanoid pathways in *ref8,* further demonstrating the central role MED5 plays in responding to cellular cues linked to phenylpropanoid metabolism.[10,14]

The *ref4-3* mutation is a missense mutation in which a conserved glycine has been mutated to a serine. This mutation leads to reduced steady-state transcript levels of several phenylpropanoid biosynthetic genes.[13] To identify other proteins and pathways that contribute to the phenotypes of *ref4-3* plants, we performed a suppressor screen for mutations that rescue the growth or metabolism of *ref4-3* plants. The suppressors we isolated display an interesting combination of growth and metabolic restoration. To identify candidate *ref four suppressor* (*rfs*) mutations, we used whole-genome sequencing and a candidate gene approach to identify intragenic mutations in *MED5a* and mutations in other Mediator tail subunit-encoding genes. Five of the suppressors identified contained intragenic missense mutations suggesting that they eliminate *ref4-3* function by disrupting folding or stability of

Table I. Mutations identified in *ref4-3* suppressors.

Line	Gene	Nucleotide Substitution	Amino Acid Substitution
rfs2-2	MED23	G:A	W129*
rfs3-3	MED5a	C:T	P917S
rfs3-4	MED5a	C:T	P917S
rfs6-2	MED5a	G:A	E1312K
rfs7-3	MED16	G:A	W898*
rfs7-4	MED2	C:T	W48*
rfs8-2	MED5a	C:T	P919L
rfs8-3	MED5a	C:T	F811L
rfs8-5	MED16	G:A	W898*
rfs8-6	MED5a	C:T	F811L
rfs9-4	MED23	G:A	W259*
rfs28-2	MED23	G:A	Splice site, intron 8
rfs31-1	MED5a	G:A	Splice site, intron 2
rfs33-2	MED5a	G:A	R387H

the protein; however, it is also possible that they reduce *ref4-3* association with Mediator, or reduce binding to other proteins, such as repressive phenylpropanoid transcription factors, required for *ref4-3* function (Table I). Preliminary data on one particularly interesting intragenic suppressor (*rfs33-2*) indicates that it is restored in lignin content but not in soluble metabolite accumulation, suggesting that these two process can be genetically disentangled. In addition to the intragenic suppressors, our candidate gene approach identified six suppressors that have nonsense mutations in the Mediator tail subunits MED2, MED16 and MED23 (Table I). Identification of mutations in these subunits as suppressors of *ref4-3* is consistent with functional and structural interactions identified in yeast and metazoans. In animals, MED5, MED16 and MED23 have been shown to form a submodule, and in yeast, loss of MED16 results in loss of MED5 and MED2 from the complex (yeast does not possess a MED23 homolog).[15,16,17] In Arabidopsis, *MED2* and *MED16* were shown to have overlapping roles in cold-induced gene expression and *MED5* and *MED16* were both shown to be required for the induction of several dark-induced genes. To evaluate the possible

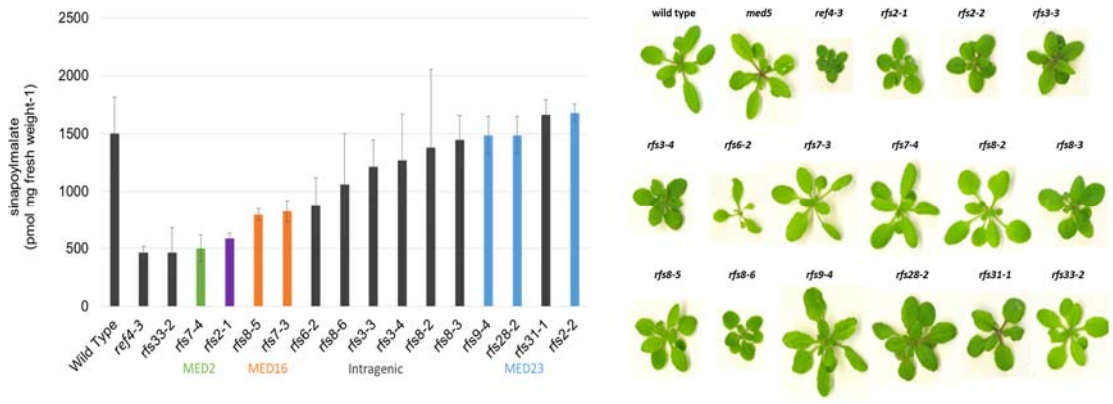

Figure 4. Whole genome sequencing identified intragenic suppressor mutations as well as Mediator subunit suppressors. Left, HPLC quantification of sinapoylmalate in *ref4-3* suppressors; right, growth characteristics of *ref4-3* suppressors.

trivial explanation that any defect in a Mediator tail subunit suppresses *ref4-3*, we generated *ref4-3 med3* and *ref4-3 med24* double mutants. These double mutant combinations do not lead to suppression of the *ref4-3* phenotype, suggesting that the suppression observed with *med5*, *med16* and *med23* mutations is specific and informative.

Although for both *MED16* and *MED23* we have independent alleles that show comparable suppressor phenotypes, we have also crossed *med* T-DNA (knockout) lines to *ref4-3*, and have independently confirmed their ability to restore growth and soluble metabolite accumulation in the *ref4-3* mutant background. For the suppressors for which we had multiple alleles (*MED16* and *MED23*), we also performed a complementation test and found that the suppressors did not complement one another (growth and metabolism was still restored in F1 plants), strong evidence that the suppressor mutations are indeed in *MED16* and *MED23.* Soluble metabolite analysis and lignin quantification of the suppressors and their respective *med* knockout lines have shown that *med23* is the strongest *ref4-3* suppressor, followed by *med16* and then *med2* (Figure 4). We have subsequently conducted a similar suppressor screen starting with the *ref4-1* mutant and have isolated 13 independent suppressors. Using these mutants, we have developed a novel Mi-Seq-based candidate gene sequencing approach to quickly and inexpensively identify mutations in Mediator subunit genes already known to suppress *ref4-3*. To date, four of these mutants have been processed in this way and *rfs21-9* has been shown to carry a premature stop codon in *MED23*.

The CDK8 kinase module of Mediator has been shown to interact with other Mediator subunits in yeast and humans where it is known to act as a repressive component.[18,19] Given that the Arabidopsis MED5 subunit represses phenylpropanoid pathway gene expression, we tested the hypothesis that this repression requires CDK8. CDK8 knockout lines (*cdk8-1*) were crossed with *ref4-3*, and the levels of sinapoylmalate were evaluated by HPLC. In *ref4-3 cdk8-1* plants the soluble metabolites and total lignin content are as low as those in *ref4-3*, yet the growth defect phenotypes in *ref4-3*, including rosette size and plant height, are largely rescued (Figure 5). These findings provide further evidence that we can

Figure 5: A *cdk8* mutation rescues the growth defect of *ref4-3*. Wild type, *cdk8-1*, *ref4-3*, *ref4-3* X *ref4-3 cdk8-1* F1 plant and *ref4-3 cdk8-1 double* mutants were compared at 4.5 weeks of age.

disentangle from one another the phenotypes seen in *ref4-3* and suggest that dwarfing in *ref4-3* plants may primarily be the result of aberrant gene expression as it is in *ref8*, rather than the direct result of perturbation of lignin deposition.[10]

4. Profiling of metabolites altered by LMID and identification of new pathways

To understand the biochemical consequences of lignin pathway perturbations, metabolomics through LC-MS analysis is being applied with the aim of characterizing and identifying compounds that are differentially abundant in mutants. A well-organized workflow is essential to process the large amounts of LC-MS data generated for each comparative profiling experiment and must include the fast and accurate recording of raw data, aligning and statistically processing data from a large number of biological replicates, and the characterization and identification of compounds. The infrastructure for acquiring accurate raw data exists, but so far, no high-throughput workflow is available. To develop such a workflow, we evaluated software packages for metabolomics, and based on accuracy, reproducibility and processing time, Progenesis QI was purchased as processing software for the metabolomics pipeline. This software has the benefit of data processing reproducibility and good data size reducing capacities, so that the most informative subset of data can be focused upon. A second major benefit of the software package is that it allows for high throughput database searching by using retention time, mass, and MS/MS data to enable more accurate characterizations and identifications of compounds. Library screening options of online databases was already available in Progenesis QI, but now communication between different software packages used for generation of home-made libraries like Instan Jchem

and NIST MSsearch has been enabled. In contrast with online databases (which only contain a small portion of all plant-specific small molecules), the creation of plant-specific libraries will contribute significantly to a more accurate identification/characterization process and enhance the total workflow. We have now developed a metabolomics pipeline that enables us to perform automated high-throughput metabolomics for LMID experiments and build plant-specific compound libraries for high-throughput characterization/identification of new secondary metabolites in plants.

We have synthesized and authenticated, in monocots, a whole range of combinatorial metabolites involving the tricin that was only recently discovered in monocot lignins.[20,21] In a paper just accepted, we describe the use of the recently developed Candidate Substrate Product Pair (CSPP) algorithm applied to Ultra-High-Performance Liquid Chromatography and Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry (UHPLC-FT-ICR-MS).[22-27] Twelve tricin-oligolignols (Figure 6) (each with up to eight isomers), including those derived from the various monolignol acetate and *p*-coumarate conjugates, were observed in maize extracts and authenticated by comparisons with the set of synthetic tricin-oligolignol dimeric and trimeric compounds. Chiral chromatography of tricin-(4'–*O*–β)-*p*-coumaryl alcohol **3H** and tricin-(4'–*O*–β)-coniferyl alcohol **3G** coupling products showed that the flavonolignols are fully racemic. The above findings provide compelling new evidence: 1) for the natural cross-coupling of tricin with monolignols and monolignol conjugates into flavonolignol dimers *in planta*; 2) that such dimers undergo further endwise coupling with additional monomers to form oligomers that are destined for lignin polymers in which chains are started by tricin; and 3) for the combinatorial nature of lignification, i.e., supporting the theory that lignin polymers are formed by combinatorial radical coupling chemistry independent of proteinaceous control.

The identification of such compounds therefore helps establish that tricin is an important monomer in the lignification of monocots, acting as a nucleation site for starting lignin chains. The array of tricin-containing products provides further evidence for the combinatorial coupling model of general lignification and supports evolving paradigms for the unique nature of lignification in monocots. In the coming year, we begin exploring the effects of perturbation of the tricin pathway on tricin-oligolignols and tricin-lignins.

Based on these synthesized compounds, an impressive series of tricin oligolignols, including many stereomers, was catalogued in lignifying internodes of maize (Table II, Table III). This work is setting us up to more completely analyze the barley lines that are being generated in this project. In support of metabolite profiling activities, we have been engaged in syntheses of important reactants, products, and metabolites to increase the ability to fully authenticate an ever more diverse array of plant metabolites. First, we synthesized a series of doubly-^{13}C-labeled hydroxycinnamic acids to aid in pathway delineation that was used to understand vacuolar sequestering.[27]

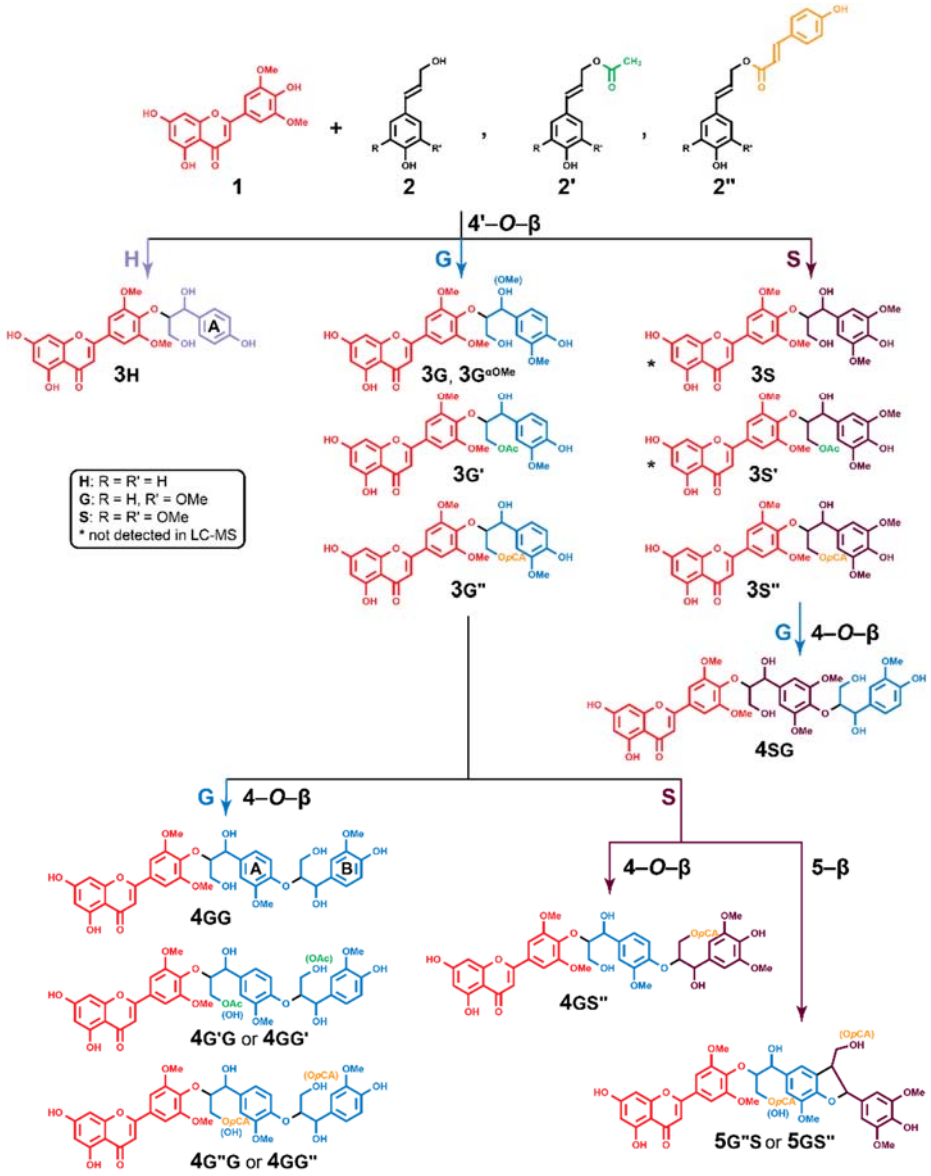

Figure 6: Tricin **1** and its oxidative coupling with monolignols **2** and monolignol conjugates **2'** and **2"** to produce tricin-oligolignols **3** (dimers), **4** (trimers), and **5** (trimers). Primes are used to indicate the acylation of monolignols and derived units in the polymer, and small upper-case letters H, G, and S, are used to designate the *p*-hydroxyphenyl, guaiacyl, or syringyl nature of the aromatic rings (and therefore the moiety's derivation from its monolignol, *p*-coumaryl, coniferyl, or sinapyl alcohol); we also refer to the A and B ring, as shown, in trimers **4**. For example, the hypothetical compound formed by coupling of coniferyl acetate **2G'** with tricin, followed by further chain extension by coupling the product dimer with sinapyl *p*-coumarate **2S"** would be designated as **4G'S"**. The two structures marked with asterisks were synthesized authentic compounds that were not found among the maize metabolites.

Table II. Tricin-dimers detected in maize.

#	t_R	m/z	Formula	Δppm	Shorthand Name	Elucidation level[a]
Dimers						
3H	19.9	495.12891	$C_{26}H_{23}O_{10}$	-1.5	T-(4–*O*–β*t*)-H	Identified
3H	20.6	495.12907	$C_{26}H_{23}O_{10}$	-1.2	T-(4–*O*–β*e*)-H	Identified
3G	20.4	525.13906	$C_{27}H_{25}O_{11}$	-2.2	T-(4–*O*–β*t*)-G	Identified
3G	21.2	525.13889	$C_{27}H_{25}O_{11}$	-2.6	T-(4–*O*–β*e*)-G	Identified
3G'	24.7	567.14943	$C_{29}H_{27}O_{12}$	-2.4	T-(4–*O*–β*t*)-G'	Identified
3G'	25.0	567.15031	$C_{29}H_{27}O_{12}$	-0.8	T-(4–*O*–β*e*)-G'	Identified
3G"	25.7	671.17480	$C_{36}H_{31}O_{13}$	-3.3	T-(4–*O*–β*t*)-G"	Identified
3G"	25.9	671.17447	$C_{36}H_{31}O_{13}$	-3.8	T-(4–*O*–β*e*)-G"	Identified
3S"	25.5	701.18628	$C_{37}H_{33}O_{14}$	-1.9	T-(4–*O*–β*t*)-S"	Identified
3S"	25.7	701.18532	$C_{37}H_{33}O_{14}$	-3.2	T-(4–*O*–β*e*)-S"	Identified
3G$^{\alpha OMe}$	24.7	539.15522	$C_{28}H_{27}O_{11}$	-1.2	T-(4–*O*–β)-G$^{\alpha OMe}$	Annotated

[a] 'Identified' indicates a structure confirmed by comparison with the synthesized authentic compound; 'Annotated' indicates a rather firm structural elucidation based on comparison of the MS^n spectrum with those of identified structural analogs and on the accurate *m/z* value; 'Characterized' indicates a fairly high degree of certainty in the structural elucidation that is based on the MS^n spectral interpretation, and accurate *m/z* value, and data available from literature and public databases.

In addition to the more common oligolignols, we have continued synthesizing a range of tetramers that involve cross-coupling that arises only from preformed dimers and higher oligomers (Figure 7) that are likely to constitute some of the products that are currently unassigned in the metabolite profile and for which we need NMR data. Such compounds are particularly important for resolving certain controversies in the lignin area such as the veracity of the so-called 4–O–5-linked units that had never before been seen in NMR spectra (but show up, rather prevalently, in various degradative methods), and the nature of lignin branching. A paper detailing how such structures are now authenticated in softwood lignins has just been accepted.[28] Also, the two 5-5-coupled compounds have literally last week been discovered now in softwood metabolite profiles.

Table III. Tricin-trimers detected in maize.

#	t_R	m/z	Formula	Δppm	Shorthand Name	Elucidation level[a]
Trimers						
4GG	18.9	721.21084	$C_{37}H_{37}O_{15}$	-4.1	T-(4–*O*–β)-G-(4–*O*–β)-G	Annotated
4GG	19.2	721.21151	$C_{37}H_{37}O_{15}$	-3.2	T-(4–*O*–β)-G-(4–*O*–β)-G	Annotated
4GG	19.4	721.21334	$C_{37}H_{37}O_{15}$	-0.6	T-(4–*O*–β)-G-(4–*O*–β)-G	Annotated
4GG	19.6	721.21185	$C_{37}H_{37}O_{15}$	-2.7	T-(4–*O*–β)-G-(4–*O*–β)-G	Characterized
4GG	19.8	721.21251	$C_{37}H_{37}O_{15}$	-1.8	T-(4–*O*–β)-G-(4–*O*–β)-G	Annotated
4GG	20.0	721.21093	$C_{37}H_{37}O_{15}$	-4	T-(4–*O*–β)-G-(4–*O*–β)-G	Annotated
4SG	19.8	751.22302	$C_{38}H_{39}O_{16}$	-1.8	T-(4–*O*–β*t*)-S-(4–*O*–β*t*)-G	Identified
4SG	20.3	751.22338	$C_{38}H_{39}O_{16}$	-1.3	T-(4–*O*–β*e*)-S-(4–*O*–β*t*)-G	Identified
4SG	20.6	751.22316	$C_{38}H_{39}O_{16}$	-1.6	T-(4–*O*–β)-S-(4–*O*–β)-G	Characterized
4SG	21.0	751.22285	$C_{38}H_{39}O_{16}$	-2	T-(4–*O*–β)-S-(4–*O*–β)-G	Characterized
4GG'	22.7	763.22264	$C_{39}H_{39}O_{16}$	-2.3	T-(4–*O*–β)-G-(4–*O*–β)-G'	Annotated
4G'G	23.2	763.22198	$C_{39}H_{39}O_{16}$	-3.1	T-(4–*O*–β)-G'-(4–*O*–β)-G	Annotated
4G'G	23.5	763.22264	$C_{39}H_{39}O_{16}$	-2.2	T-(4–*O*–β)-G'-(4–*O*–β)-G	Annotated
4G'G	24.0	763.22147	$C_{39}H_{39}O_{16}$	-3.8	T-(4–*O*–β)-G'-(4–*O*–β)-G	Annotated
5GS"	24.3	879.24808	$C_{47}H_{43}O_{17}$	-2.8	T-(4–*O*–β)-G-(5–β)-S"	Characterized/Annotated
5GS" [5G"S]	25.2	879.24942	$C_{47}H_{43}O_{17}$	-1.3	T-(4–*O*–β)-G-(5–β)-S" [T-(4–*O*–β)-G"-(5–β)-S]	Characterized
5GS"	25.7	879.24975	$C_{47}H_{43}O_{17}$	-0.9	T-(4–*O*–β)-G-(5–β)-S"	Characterized/Annotated
5GS" [5G"S]	26.3	879.24928	$C_{47}H_{43}O_{17}$	-1.5	T-(4–*O*–β)-G-(5–β)-S" [T-(4–*O*–β)-G"-(5–β)-S]	Characterized
5GS" [5G"S]	26.9	879.24915	$C_{47}H_{43}O_{17}$	-1.6	T-(4–*O*–β)-G-(5–β)-S" [T-(4–*O*–β)-G"-(5–β)-S]	Characterized
5GS" [5G"S]	27.4	879.24823	$C_{47}H_{43}O_{17}$	-2.7	T-(4–*O*–β)-G-(5–β)-S" [T-(4–*O*–β)-G"-(5–β)-S]	Characterized
4G"G [4GG"]	24.8	867.24907	$C_{46}H_{43}O_{17}$	-1.7	T-(4–*O*–β)-G"-(4–*O*–β)-G [T-(4–*O*–β)-G-(4–*O*–β)-G"]	Characterized
4G"G [4GG"]	25.0	867.24993	$C_{46}H_{43}O_{17}$	-0.7	T-(4–*O*–β)-G"-(4–*O*–β)-G [T-(4–*O*–β)-G-(4–*O*–β)-G"]	Characterized
4G"G [4GG"]	25.7	867.24982	$C_{46}H_{43}O_{17}$	-0.9	T-(4–*O*–β)-G"-(4–*O*–β)-G [T-(4–*O*–β)-G-(4–*O*–β)-G"]	Annotated
4GG"	26.0	867.24835	$C_{46}H_{43}O_{17}$	-2.6	T-(4–*O*–β)-G-(4–*O*–β)-G"	Annotated
4G"G [4GG"]	26.3	867.24938	$C_{46}H_{43}O_{17}$	-1.4	T-(4–*O*–β)-G"-(4–*O*–β)-G / T-(4–*O*–β)-G-(4–*O*–β)-G"	Characterized
4G"G [4GG"]	26.5	867.24895	$C_{46}H_{43}O_{17}$	-1.9	T-(4–*O*–β)-G"-(4–*O*–β)-G / T-(4–*O*–β)-G-(4–*O*–β)-G"	Characterized
4GS"	26.2	897.25787	$C_{47}H_{45}O_{18}$	-3.6	T-(4–*O*–β)-G-(4–*O*–β)-S"	Annotated
4GS"	26.4	897.25903	$C_{47}H_{45}O_{18}$	-2.3	T-(4–*O*–β)-G-(4–*O*–β)-S"	Annotated
4GS"	26.5	897.25803	$C_{47}H_{45}O_{18}$	-3.5	T-(4–*O*–β)-G-(4–*O*–β)-S"	Annotated
4GS"	26.7	897.25813	$C_{47}H_{45}O_{18}$	-3.4	T-(4–*O*–β)-G-(4–*O*–β)-S"	Annotated
4GS"	26.9	897.25816	$C_{47}H_{45}O_{18}$	-3.3	T-(4–*O*–β)-G-(4–*O*–β)-S"	Annotated

[a] The meaning of 'Identified' and 'Characterized' are described Table II.

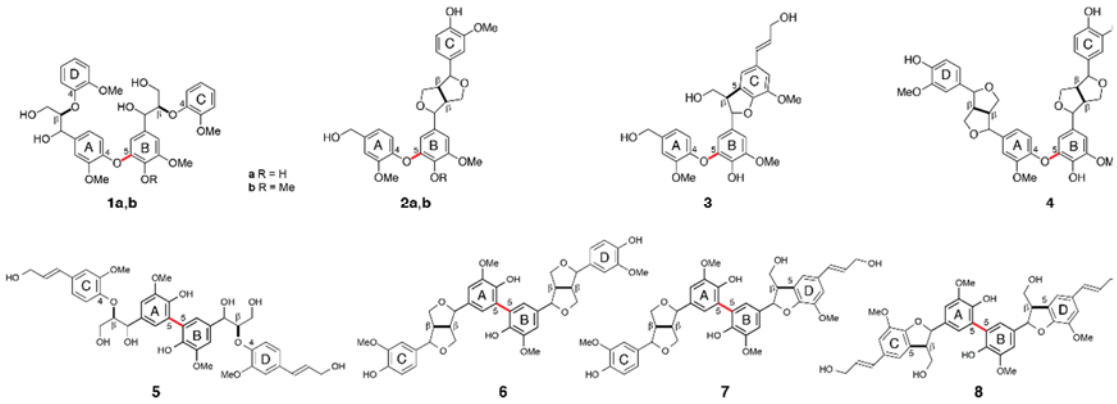

Figure 7: Newly synthesized complex oligolignols for NMR, metabolite profiling and compound authentication. Compounds **1**-**4** contain 4–O–5-coupled units, whereas compounds **5**-**8** contain 5–5-coupled units.

5. Determine whether LMID is caused by accumulation of phenolic molecules with hormone activity

The growth defects observed in plants affected in lignification could be a direct consequence of accumulating intermediates of the phenylpropanoid pathway.[29] There has been a long standing discussion on the possible bioactivity of phenylpropanoids and/or simple phenylpropanoid-coupled molecules, and for this project we tested the impact of such compounds on cell division activity of Arabidopsis cells.[30] *Arabidopsis thaliana* leaf mesophyll protoplasts expressing a histone H2B-YFP fusion protein were seeded into micro-well plates and the mitotic rate of individual protoplasts was followed through time lapse imaging with a confocal microscope following treatment with several compounds. Among the 82 compounds tested were selected phenylpropanoids and phenylpropanoid-derived compounds and described inhibitors of the phenylpropanoid pathway to mimic lignin pathway perturbations. In addition, a set of putative inhibitors for lignification was also used.[31,32] This set of compounds was the outcome of an in-house chemical genetics screen for inhibitors of lignin deposition in Arabidopsis. In an initial screen, both stimulators and inhibitors of cell proliferation were detected among the compounds tested. However, results turned out not to be reproducible in follow-up experiments. Nevertheless, based on several independent experiments, a set of nine compounds were selected for a more profound analysis in which the compounds were tested at two different concentrations. However, none of the selected compounds stimulated cell division activity under the conditions tested.

As the initial approach to study bioactivity of phenolic compounds suffered from a lack of reproducibility, it was decided to shift towards a more targeted approach by focusing on phenylpropanoids known to accumulate in a *c4h* mutant. Knocking-out *C4H* is lethal to the plant but knock-downs can survive. Compounds that accumulate due to C4H inhibition might have bioactivity. One of the interesting compounds accumulating in this mutant is cinnamoyl malate, derived from *trans*-cinnamic acid (CA), the direct substrate of C4H.[33] These data suggest that CA also accumulates in *c4h* mutants. Its photo-isomerization product (*cis*-CA) is

only detected in trace amounts in plants.[34,35] An adequate explanation concerning the molecular mechanism by which CA affects plant growth and development is lacking so far. We re-evaluated the role of both isomers as natural regulators of plant development and showed that *trans*-CA is a biologically inactive compound, while its *cis*-isomer is biologically active. The morphological defects caused by *cis*-CA were studied in detail using root development as a model. *cis*-CA increased the frequency of lateral roots. Although these effects resemble to some extent the physiological effects caused by perturbed auxin or ethylene homeostasis, the exact molecular mechanism is not known. To find direct support for a crosstalk between *cis*-CA and auxin signaling, we monitored whether *cis*-CA could affect the nuclear auxin response using the auxin reporter line *DR5rev:GFP*. Here, considerable similarity was found between *cis*-CA and NAA treatment in the *DR5*-driven GFP expression line. Similar results were obtained using the DR5:LUC and DII-VENUS reporter lines.[36,37] Together, these results show that *cis*-CA has auxin-like effects on plant development and affects the spatial distribution of the auxin response at low micro molar concentrations.

In parallel we tried to increase endogenous CA levels using piperonylic acid (PA) and methylenedioxycinnamic acid (MDCA) to perturb the pathway at the level of C4H and 4CL respectively. Interestingly, the phenotype of Arabidopsis plants treated with PA and MDCA were different. Whereas MDCA treatment phenocopied to some extent the phenotype of previous described CA treated plants, PA induced adventitious roots rather than lateral roots. Based on the similarity in phenotype of MDCA and CA treated plants, we decided to focus on this inhibitor. To obtain profound insight into the effect of MDCA on plant growth and development, plants were treated with different MDCA concentrations (0 to 40 μM). Twelve days after germination, both the primary root and rosette growth of the seedlings were analyzed. Compared to the control, the MDCA-treated plants had more lateral roots and the roots displayed a marked agravitropic response. In addition, a dose-dependent decrease of both the primary root length and leaf area was observed. In contrast to these inhibitory effects MDCA stimulated lateral root formation and adventitious rooting in a dose-dependent manner.

Previous *in vitro* assays based on heterologous systems provided evidence that MDCA acts as a competitive inhibitor of 4CL.[32,38] As this enzyme catalyzes a key step of the general phenylpropanoid pathway, we used phenolic profiling to evaluate how the carbon flux over this pathway is redirected in seedlings upon treatment with MDCA. To this end the methanol-soluble metabolites of twelve day-old *in vitro* grown Arabidopsis seedlings treated with 10 μM MDCA were analyzed by UPLC-MS. A total of 1247 peaks were detected over the whole experiment. Principal component analysis revealed that MDCA-treated samples separated from control samples based on a combination of the first and second principal component, indicating metabolic shifts upon MDCA treatment. Screening the top list of highest accumulating compounds in MDCA treated plants revealed active MDCA processing. In addition conjugates of CA were detected. Although no trace of *cis*-CA was found in the plant extracts, the increase in CA conjugates indicates that our strategy to increase *in planta* CA concentrations has been successful. The top 10 list of compounds for which the concentration dropped upon MDCA treatment holds several glycosylated forms of

ferulic acid-containing phenolic dimers, glucosinolates and flavonoids. The observed shift in the phenolic profile of MDCA-treated seedlings is in line with the inhibition of an enzyme catalyzing an early step in the core phenylpropanoid pathway. The MDCA-induced reduction of glycosylated oligolignols is considered indicative of a drop in lignin deposition.[39] To provide supporting evidence for this assumption we showed the inhibitory effect of MDCA on the formation of the lignin rich Casparian strip in the main root.[40] The formation of the Casparian strip was restored by supplying MDCA-treated plants with a mixture of coniferyl and sinapyl alcohol, confirming that MDCA affects the biosynthesis of lignin. The MDCA-characteristic phenotypes such as the reduction of the primary root length and the proliferation of lateral roots could not be restored by adding monolignols indicating that the reduced lignin deposition in Arabidopsis seedlings is not at the basis of the observed developmental defects caused by MDCA. We are currently studying whether CA could be involved in the MDCA mediated phenotype.

6. Understanding lignin structure and discovery of unknown pathways using novel tools

Disruption of the genes encoding both cinnamyl alcohol dehydrogenases (CADs), including CADC and CADD, in Arabidopsis, results in the atypical incorporation of hydroxycinnamaldehydes into lignin. Another strategy to change lignin composition is downregulation or overexpression of ferulate 5-hydroxylase (F5H), which results in lignins enriched in guaiacyl or syringyl units, respectively. We combined these approaches to generate plants enriched in coniferaldehyde-derived lignin units or lignins derived primarily from sinapaldehyde. The *cadc cadd* and *fah1 cadc cadd* plants are similar in growth to wild-type plants even though their lignin compositions are drastically altered. In contrast, disruption of CAD in the F5H-overexpressing background results in dwarfism. The dwarfed phenotype observed in these plants does not appear to be related to collapsed xylem, a hallmark of many other lignin-deficient dwarf mutants. *cadc cadd*, *fah1 cadc cadd*, and *cadd F5H* overexpressing plants have increased enzyme-catalyzed cell wall digestibility. Given that these CAD-deficient plants have similar total lignin contents and only differ in the amounts of hydroxycinnamaldehyde monomer incorporation, these results suggest that hydroxycinnamaldehyde content is a more important determinant of digestibility than lignin content. This work was particularly successful, resulting in a publication in The Plant Cell.[41] The next step (in the current year) is to determine if the dwarfed lines can be rescued by the methods currently developed here.

Work is progressing on extending our ‘zip-lignin’ work that previously had to remain independent of our GCEP projects but now logically falls under it.[42,43] In the initial work, we developed methods to introduce readily cleavable ester linkages into the backbone of the lignin polymer by augmenting plants with *FMT* (feruloyl-CoA:monolignol transferase) genes that allowed them to produce monolignol ferulate conjugates; these conjugates were shown to have been successfully biosynthesized in poplar, transported to the wall, and incorporated into the lignin polymer. As a result, the digestibility following mild alkaline pretreatments, as well as pulping performance were significantly improved.[43]

In work under separate DOE funding, we have significantly improved the methodology used to authenticate monolignol ferulate incorporation into lignins. We now use MRM

methods on a triple-quad mass spectrometer (coupled to an HPLC) and have synthesized isotopically labeled variants of the products to allow accurate quantification.

7. Scalability via translation to energy crops via barley and ultimately poplar

The aim of this task in the project is to generate crop plants suitable for bioenergy use. To this end, genes identified in previous studies as important for saccharification or LMID will be identified in barley and poplar, and plants deficient in these genes will be generated. We are utilizing the relatively new CRISPR/Cas9 technology to generate targeted gene knock-outs in barley. This system, in which a designed guide RNA (gRNA) targets the Cas9 endonuclease to the desired gene, generates a DNA double strand break at the targeted site. DNA repair mechanisms will then repair the break in the DNA, but in the process, will often generate small insertions or deletions in the gene sequence, disrupting gene function.[44] This system has already been used to generate gene knock-outs in crop plants such as wheat and rice, and we have already used it to target several genes in barley in this project.[45,46] This system is advantageous to RNAi because complete knock-outs are readily generated in the CRISPR system, while RNAi reduces gene expression, but rarely completely. Therefore, we are in the process of generating several gene knock-outs in the lignin pathway in barley, including lignin genes and shikimate pathway genes.

In pursuit of this goal, we have targeted five barley genes implicated in the biosynthesis of monolignols for knock-out using the CRISPR system. CRISPR gRNAs were designed against the lignin biosynthesis genes *C4H1, 4CL1,* and *CCR1*. Our choice was based on gene expression profiles over 16 barley tissues. Previous work using RNAi lines for these genes has demonstrated reductions in lignin content with little or no effect on barley plant stature. This is in contrast to *Arabidopsis*, where plants defective in these genes have severe growth

Table IV: Summary of mutation detection in CRISPR-targeted genes

Gene	Plants Tested	Indels Detected	Sequencing Confirmed	Confirmed Insertions	Confirmed Deletions
HvC4H1	134	65	11	1	10
HvHSR1	64	6	6	4	2
HvLMR1	121	3	3	0	3
HvCCR1	54	3	3	1	2
Hv4CL1	94	7	7	6	1

phenotypes.[33,47,48] The lack of a growth phenotype may be due to gene redundancy in barley or could be due to incomplete reduction of gene function from RNAi. We have also targeted the barley homologs of the *LMR1* and *HSR1* (presented as mutant 16-91 in past reports) genes, and all of the targeted genes have relatively high expression in lignifying tissues like internodes.[49] gRNAs were designed against the above genes and cloned into a pBRACT214 vector modified to express CAS9 along with the gRNA under control of the constitutive Ubi promoter. These constructs were then transformed into barley (cv. Golden Promise) using an *Agrobacterium*-mediated protocol.[50]

Primary transformants (T_0) were screened for mutations using Indel Detection by Amplicon Analysis (IDAA). In this method, primers flanking the CRISPR-targeted site amplify a fragment of DNA containing any generated mutations. One primer contains a fluorescent tag, allowing for precise size detection when run using DNA capillary electrophoresis (+/- 1 bp).[51] By using this technique, we have identified at least three mutations in each of the five genes targeted (Table IV).

Mutations of interest detected by IDAA were confirmed using Sanger DNA sequencing. Deletions ranged from 1 to 25 bp, while all but one insertion mutation was of a single nucleotide. It is not immediately clear why the gRNA designed against *HvC4H1* is much more effective at generating mutations than the other constructs, but GC-content, sequence context and RNA secondary structure have all been implicated in affecting CRISPR effectiveness.[52] Plants with confirmed mutations have been grown and progeny from this initial generation collected. These progeny will be screened to obtain homozygous mutants that lack the gRNA-Cas9 construct (to prevent ongoing mutation at the targeted sites). These homozygotes will be assessed on their cell wall and growth properties, and to their suitability for improved biofuel production.

We have previously generated a set of RNAi knock-downs of many monolignol biosynthetic genes in barley. These were generated outside of GCEP, but also now logically fall under this project. The lignin composition of these RNAi lines has been preliminarily structurally characterized by NMR in the Ralph lab. There is an excellent range of lignin compositions with wide ranges in H, G, and S, as well as *p*-coumarate (*p*CA) and tricin levels. Such extremes, coupled with the work on the agronomic state of these plants, provides an extremely rich resource for the planned work on mitigating agronomic issues in transgenics.

To discover new processes involved or affected during lignification, we are undertaking a combined transcriptomic/metabolomic approach. We first performed a pilot study to ascertain which developmental stage would be best for discerning differences in lignin metabolites, and if differences in metabolites could be detected in two RNAi lines. First, wild-type barley plants (cv Golden Promise) were grown for up to 9 weeks in a glasshouse and both stem and leaf samples were collected when plants were 36, 43, 50, 57, and 64 days old. Each tissue sample was then ground and extracted with methanol and extracts were used to detect metabolites with UPLC-MS (Boerjan lab). In stems, metabolites involved in lignin production, particularly oligolignols, were sharply increased at 57 days (Figure 8). This led us to choosing 57 days, which corresponded to barley developmental stage Zadoks 3.9-4.1, as our targeted time point.[53] Secondly, leaves and stems collected from C3H and HCT RNAi lines were also assayed with UPLC-MS (Boerjan lab). Analysis of methanol extracts of these plants revealed an increase in abundance of several H-unit containing dilignols and a decrease in abundance in several G-unit containing di- and trilignols. This is in accordance with the NMR data that indicate high levels of H monolignols and decreases of G monolignols in both C3H and HCT RNAi plants.

These data indicated that a large scale metabolomic and transcriptomic experiment could be performed to assay which metabolites and genes are affected when lignin synthesis is perturbed. We grew five replicates of 18 RNAi lines targeted against 10 different monolignol biosynthesis enzymes along with five control lines until the majority had reached Zadoks stage 3.9-4.1. We then harvested and ground the first three internodes of each plant. The stem powder from each sample was split into two portions. One portion was extracted with methanol and used for UPLC-MS analysis, while the other portion was used to isolate RNA. This RNA was used for gene expression analysis on a 60K barley Agilent microarray chip.

Initial analysis suggests that the genes targeted with the RNAi constructs are down-regulated in the RNAi plants except the single CCoAOMT line and the C4H lines (one C4H paralog is not measured on this barley microarray). This initial analysis also showed that there are 146 genes significantly up-regulated in at least two RNAi lines when compared to

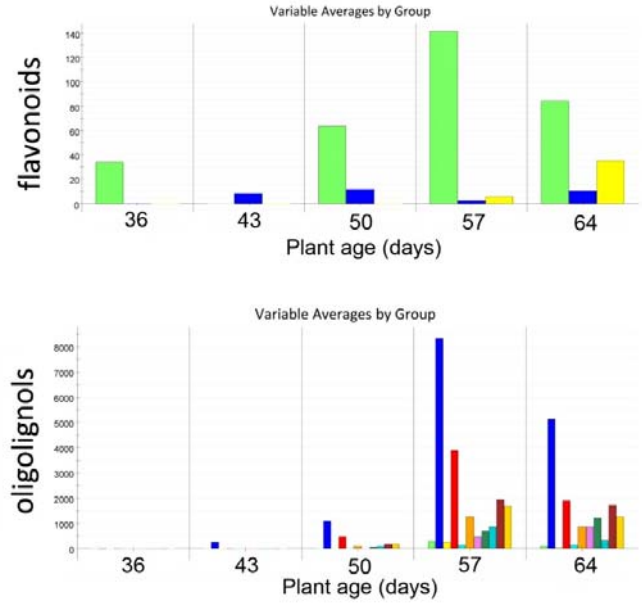

Figure 8: Relative abundance of selected metabolites in barley stems. Barley plants were grown for the time indicated, and stems were ground and extracted with methanol. Extracts were dried and re-dissolved in cyclohexane:water (1:1) and used for UPLC-MS.

wild type barley and 113 genes significantly down-regulated in at least two RNAi lines when compared to wild type barley. However, further analysis needs to be performed on this data before firm conclusions can be drawn. Furthermore, we are currently performing UPLC-MS on all RNAi lines, and this data will be combined with the microarray data to obtain a systems biology view of lignification in barley.

Progress

Table V. Progress Summary

Task	Description	Who	Prog
1	Use next generation sequencing technologies to identify the *c3h* suppressor mutations	SL	✓
2	Determine whether suppressors of LMID in *ccr1*, *c4h,* and *c3h* are known components of Mediator	CH, SL	✓
3	Investigate whether Mediator mutations can overcome LMID in *ccr1* (task underway) and *c4h* (task complete) plants as is the case for *ref4 rfr1c3h*	CC	✔
4	Depending on the results of the experiments above, the identity of the suppressor genes, and the data from metabolite profiling, develop and test alternative hypotheses regarding other mechanisms of LMID rescue	CH, SL	❍
5	Generate dexamethasone–inducible transgene constructs for *REF4* and *RFR1* using the pOpOn vector system	CC	✔
6	Transform *ref4 rfr1 c3h* mutants with these constructs and isolate several stable transgenic lines for each	CC	✔
7	Confirm that the *REF4* and *RFR1* transgenes are functional by comparison of growth and biochemical phenotypes of T1 plants in the presence and absence of dexamethasone to *c3h* and *ref4 rfr1 c3h* control plants. Plants grown in the presence of dexamethasone should phenocopy *c3h* plants	CC	❍
8	Grow plants (*ref4 rfr1 c3h* mutants containing dexamethasone-inducible REF4/RFR1) to maturity, apply dexamethasone, and monitor metabolic and morphological changes over time, to determine the optimal time point and tissue to use for gene expression analysis	CC	❍
9	Compare global gene expression of *ref4 rfr1 c3h* plants before and after the induction of RFR1 with dexamethasone, as well as non-transgenic *ref4 rfr1 c3h* control plants, using whole-genome transcriptome sequencing technology (RNA seq)	CC	❍
10	Generate dexamethasone-inducible *ref4-3* transgene constructs	CC	✔
11	Transform these transgene constructs into *ref4*- and *ref4 rfr1*-deficient plants	CC	✔
12	Measure gene expression changes upon application of dexamethasone using RNA seq as described above	CC	❍
13	Compare gene expression changes in *ref4 rfr1* plants upon induction of *ref4-3* with the gene expression changes seen in *ref4 rfr1 c3h* plants upon induction of RFR1. Any expression changes identified in both experiments will be considered strong candidates for Mediator targets responsible for dwarfing	CC	❍
14	Develop comparative CSPP networks for mutants with LMID and LMID suppressor mutants	WB	✓
15	Identify metabolites that differ between mutants with LMID and the corresponding suppressors by CSPP and lignomics	WB, JR	❍
16	Purify differentially accumulated compounds	WB	❍
17	Analyze purified compounds by NMR for structural elucidation	JR	❍
18	Chemically synthesize purified compounds for authentication by UPLC-MS	WB, JR	✓
19	Collect aromatic compounds relevant to the consequences of pathway perturbations. Over 200 authentic compounds are already in our 'library'	WB, JR	✓
20	Assay the purified compounds from 13task 16 and those of task 19 for cell division-promoting activity in the HCS-platform	WB	✓
21	Select molecules with cell division-promoting activity	WB	✓
22	Compare the structures of the cell division-promoting compounds for common chemical entities	WB, JR	✓
23	Apply these molecules to *in vitro*-grown plants transformed with reporter gene constructs that respond to the different plant hormones (P*DR5-GUS* and P*ARR5-GUS*)	WB	✓

Legend: ✓= partially completed; ✔= completed; ❍= in progress.

Publications and Patents

Publications (past year)

1. Lan W., Morreel K., Lu F., Rencoret J., del Rio J.C., Voorend W., Vermerris W., Boerjan W., and Ralph J. Maize tricin-oligolignol metabolites and their implications for monocot lignification. *Plant Physiol*. 171, in press, (2016).
2. Rinaldi R., Jastrzebshi R., Clough M.T., Ralph J., Kennema M., Bruijnincx P.C.A., Weckhuysen B.M. Paving the way for lignin valorisation: Recent advances in bioengineering, biorefining and catalysis. *Angewandte Chemie* (English Edition): in press, (2016).
3. Yue F., Lu F., Ralph S.A., Ralph J. Identification of 4–O–5-units in softwood lignins via definitive lignin models and NMR. *Biomacromolecules*: in press, (2016).
4. Mottiar Y., Vanholme R., Boerjan W., Ralph J., Mansfield S.D. Designer lignins: harnessing the plasticity of lignification. *Current Opin. Biotechnol*. 37, 190-200, (2016).
5. Anderson, N. A., Tobimatsu, Y., Ciesielski, P. N., Ximenes, E., Ralph, J., Donohoe, B. S., Ladisch, M., and Chapple C. Manipulation of guaiacyl and syringyl monomer synthesis in *cad-c cad-d* Arabidopsis results in atypical lignin biosynthesis and modified cell wall structure. *Plant Cell* 27, 2195-2209, (2015).
6. Kim, J. I., Dolan, W. L. Anderson N. A., and Chapple, C. Indole glucosinolate biosynthesis limits phenylpropanoid accumulation in *Arabidopsis thaliana*. *Plant Cell*, 27, 1529-1546, (2015).
7. Grabber, J.H., Santoro, N., Foster, C.E., Elumalai, S., Ralph, J., and Pan, X. Incorporation of flavonoid derivatives or pentagalloyl glucose into lignin enhances cell wall saccharification following mild alkaline or acidic pretreatments. *BioEnergy Research* 8, 1391-1400 (2015).
8. Kim, J.I., Ciesielski, P.N., Donohoe, B.S., Chapple, C., and Li, X. Chemically induced conditional rescue of the *reduced epidermal fluorescence8* mutant of Arabidopsis reveals rapid restoration of growth and selective turnover of secondary metabolite pools. *Plant Physiol*., 164, 584-595, (2014).

Previous Publications

1. Bao, Z., Benson, S., Cui, Y., Dionne, J.A., Maher, K., Boerjan, W., Halpin, C., Nelson, R., Nichols, D., Ralph, J., and Ramakrishnan, T. S. In search of clean, affordable energy, *Oilfield Review*, 26, 4-15, (2014).
2. Bonawitz, N.D., Kim, J.I., Tobimatsu, Y., Ciesielski, P.N., Anderson, N.A., Ximenes, E., Maeda, J., Ralph, J., Donohoe, B.S., Ladisch, M., and Chapple C., Disruption of Mediator rescues the stunted growth of a lignin-deficient Arabidopsis mutant, *Nature*, 509, 376-380, (2014).
3. Dima, O., Morreel, K., Vanholme, B., Kim, H., Ralph, J., Boerjan, W. Small glycosylated lignin oligomers are stored in Arabidopsis leaf vacuoles, *Plant Cell*, 27, 695-710, (2015).
4. Morreel, K., Y. Saeys, O. Dima, F. Lu, Y. Van de Peer, R. Vanholme, J. Ralph, B. Vanholme, W. Boerjan, Systematic structural characterization of metabolites in Arabidopsis via candidate substrate-product pair networks, *Plant Cell*, 26, 929-945, (2014).
5. Sundin, L., Vanholme, R., Geerinck, J., Goeminne, G., Höfer, R., Kim, H., Ralph, J., and Boerjan W. Mutation of the inducible ARABIDOPSIS THALIANA CYTOCHROME P450 REDUCTASE2 alters lignin composition and improves saccharification, *Plant Physiol*., 166, 1956-1971, (2014).
6. Tobimatsu, Y., Wagner, A., Donaldson, L., Mitra, P., Niculaes, C., Dima, O., Kim, J.I., Anderson, N., Loqué, D., Boerjan, W., Chapple, C., and Ralph J. Visualization of plant cell wall lignification using fluorescence-tagged monolignols, *Plant J*., 76, 357-366, (2013).
7. Tobimatsu, Y., Van de Wouwer, D., Allen, E., Kumpf, R., Vanholme, B., Boerjan, W., Ralph, J. A click chemistry strategy for visualization of plant cell wall lignification, *Chem. Commun*., 50, 12262-12265, (2014).
8. Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goeminne, G., Kim, H., Cross, J., Morreel, K., Araujo, P., Welsh, L., Haustraete, J., McClellan, C., Vanholme, B., Ralph, J., Simpson, G. G., Halpin, C., and Boerjan W. Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway, *Science*, 341, 1103-1106, (2013)

Presentations

1. Boerjan W. Genetic engineering of wood quality to improve biomass processing. Invited talk at ITQB, Lisboa, Portugal, April 2016
2. Boerjan W. Systems biology of lignification to identify new pathways and genes. Invited talk at the PhD program Plants For Life and Molecular Biosciences; module Plant Biotechnology for Sustainability and Global Economy, ITQB, Lisboa, Portugal, April, 2016
3. Boerjan W. From systems biology in Arabidopsis to translational research in poplar. Invited talk at the LBNet 1st International Conference "Construction and deconstruction of lignocellulosic biomass" Shrigley Hall, Cheshire, UK, February 2016
4. Boerjan W. Comparative metabolite profiling to discover new enzymes in phenylpropanoid biosynthesis. Talk (organiser) at the meeting Advances in Cellular Metabolomics – A Plant Perspective, Gent, Belgium, December 2015
5. Boerjan W. Engineering of lignification in poplar. Talk (organizer) at Brazil Bioeconomy autumn school, Rio de Janeiro, Brazil, May 2015
6. Chapple C. How do plants mediate crosstalk between biochemical pathways? Invited talk at Microbial and Plant Systems Modulated by Secondary Metabolites Meeting, Joint Genome Institute, May 2016.
7. Chapple C. How do plants mediate crosstalk between biochemical pathways? Invited talk at Department of Biochemistry, Michigan State University, October 2015.
8. Chapple C. Transcriptional feedback mechanisms governing lignin biosynthesis in Arabidopsis. Invited talk at Gordon Conference on Plant Cell Walls. July, 2015.
9. Halpin, C. Understanding and manipulating lignin biosynthesis to enable biomass improvement. Invited talk at the LBNet 1st International Conference "Construction and deconstruction of lignocellulosic biomass" Shrigley Hall, UK, February 2016
10. McClellan, C. Lignin engineering in barley: using Arabidopsis to identify targets. Invited DPS talk, Dundee, February 2016
11. Halpin, C. Tailoring biomass feedstocks for biorefineries. Invited talk at BBSRC Partnering Workshop, PhilRice, Science City of Munoz, Philippines, November 2015.
12. Halpin, C. Spinning straw into gold. Invited talk at the CECHR Annual Symposium, Dundee, April 2015.
13. Ralph, J. Lignin Utilization: from what we have now to where we can go with plant design. Invited talk at UK Royal Society, LBNet Workshop, Manchester, UK, May 2015.
14. Ralph, J. Plant improvement: How the future is shaping up for more facile plant cell wall conversion and improved valorization. Invited talk at USDOE BioEnergy 2015, Washington, DC, June 2015.
15. Ralph, J. Engineering lignins designed for deconstruction. Invited talk at Gordon Research Conference on Plant Metabolic Engineering, GRC: Waterville Valley, NH, July 2015.
16. Ralph, J. Pursuing plant traits for reducing processing recalcitrance and adding value. Invited talk at Gordon Research Conference on Plant Cell Walls, GRC: Bentley U., Waltham, MA, July 2015.
17. Yue, F. Synthesis of lignin-derived dimers from thioacidolysis followed by Raney nickel desulfurization and their uses as GC quantitation standards. Invited talk at American Chemical Society National Meeting, ACS: Boston, MA, August 2015.
18. Ralph, J. Lignin Utilization: What can we do with 'normal' lignins and the recently 'discovered' novel lignins? Invited talk at MARS cocoa co-product Workshop, Morristown, NJ, August 2015.
19. Ralph, J. Genetic engineering of lignins for improved process and utilization potential – Designer lignins. Marcus Wallenberg Invited Talks, Stockholm, Sweden, September 2015.
20. Ralph, J. Designer Lignins. Invited talk at International Symposium of Wood, Fiber and Pulping Chemistry, U. Vienna, BOKU.: Vienna, Austria, September 2015.
21. Ralph, J. Designer lignins. Invited talk at the 60th Lignin Symposium, Tsukuba, Japan, November 2015.
22. Ralph, J. Redesigning lignin for improved plant cell wall deconstruction – a case study. Invited talk at the Tokyo University of Agriculture and Technology Seminar Series, Koganei Campus, Tokyo University of Agriculture and Technology, Tokyo, Japan, November 2015.

23. Ralph, J. Redesigning lignin for improved plant cell wall deconstruction – a case study. Invited talk at Tokyo University of Agriculture and Technology Seminar Series, Fuchu Campus, Tokyo University of Agriculture and Technology, Tokyo, Japan, November 2015.
24. Ralph, J. 'Designing' lignins for the biorefinery. Invited talk at the International Symposium on Frontiers Research in the Sustainable Humanosphere, Kyoto U., Japan, November 2015.
25. Ralph, J. Designer lignins for improving the utility of plant biomass. Invited talk at Global Innovation Research Organization Symposium, The Global Innovation Research Organization: Koganei Campus, Tokyo U. of Agriculture and Technology, November 2015.

References

1. Marriott, P. E., R. Sibout, C. Lapierre, J. U. Fangel, W. G. T. Willats, H. Hofte, L. D. Gomez, S. J. McQueen-Mason, Range of cell-wall alterations enhance saccharification in *Brachypodium distachyon* mutants, *Proc. Natl. Acad. Sci. U.S.A.*, 111, 14601-14606, 2014.
2. Liu, Y., S. You, M. Taylor-Teeples, W. L. Li, M. Schuetz, S. M. Brady, C. J. Douglas, BEL1-LIKE HOMEODOMAIN6 and KNOTTED ARABIDOPSIS THALIANA7 interact and regulate secondary cell wall formation via repression of *REVOLUTA*, *Plant Cell*, 26, 4843-4861, 2014.
3. Van Acker, R., J.-C. Leplé, D. Aerts, V. Storme, G. Goeminne, B. Ivens, F. Légée, C. Lapierre, K. Piens, M. C. E. Van Montagu, N. Santoro, C. E. Foster, J. Ralph, W. Soetaert, G. Pilate, W. Boerjan, Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase, *Proc. Natl. Acad. Sci. U.S.A.*, 111, 845-850, 2014.
4. Vanholme, R., I. Cesarino, K. Rataj, Y. Xiao, L. Sundin, G. Goeminne, H. Kim, J. Cross, K. Morreel, P. Araujo, L. Welsh, J. Haustraete, C. McClellan, B. Vanholme, J. Ralph, G. G. Simpson, C. Halpin, W. Boerjan, Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway in *Arabidopsis*, *Science*, 341, 1103-1106, 2013.
5. Yang, F., P. Mitra, L. Zhang, L. Prak, Y. Verhertbruggen, J.-S. Kim, L. Sun, K. Zheng, K. Tang, M. Auer, H. V. Scheller, D. Loqué, Engineering secondary cell wall deposition in plants, *Plant Biotechnol*. J., 11, 325-335, 2013.
6. Kubo, M., M. Udagawa, N. Nishikubo, G. Horiguchi, M. Yamaguchi, J. Ito, T. Mimura, H. Fukuda, T. Demura, Transcription switches for protoxylem and metaxylem vessel formation, *Genes Dev.*, 19, 1855-1860, 2005.
7. Kim, J. I., P. N. Ciesielski, B. S. Donohoe, C. Chapple, X. Li, Chemically induced conditional rescue of the *reduced epidermal fluorescence8* mutant of Arabidopsis reveals rapid restoration of growth and selective turnover of secondary metabolite pools, *Plant Physiol*., 164, 584-595, 2014.
8. Michelmore, R. W., I. Paran, R. V. Kesseli, Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 9828-9832, 1991.
9. Zhao, J., W. Zhang, Y. Zhao, X. Gong, L. Guo, G. Zhu, X. Wang, Z. Gong, K. S. Schumaker, Y. Guo, SAD2, an importin β-like protein, is required for UV-B response in *Arabidopsis* by mediating MYB4 nuclear trafficking, *Plant Cell*, 19, 3805-3818, 2007.
10. Bonawitz, N. D., J. I. Kim, Y. Tobimatsu, P. N. Ciesielski, N. A. Anderson, E. Ximenes, J. Maeda, J. Ralph, B. S. Donohoe, M. Ladisch, C. Chapple, Disruption of Mediator rescues the stunted growth of a lignin-deficient *Arabidopsis* mutant, *Nature*, 509, 376-380, 2014.
11. Ruegger, M., C. Chapple, Mutations that reduce sinapoylmalate accumulation in *Arabidopsis thaliana* define loci with diverse roles in phenylpropanoid metabolism, *Genetics*, 159, 1741-1749, 2001.
12. Stout, J., E. Romero-Severson, M. O. Ruegger, C. Chapple, Semidominant mutations in *reduced epidermal fluorescence 4* reduce phenylpropanoid content in Arabidopsis, *Genetics*, 178, 2237-2251, 2008.
13. Bonawitz, N. D., W. L. Soltau, M. R. Blatchley, B. L. Powers, A. K. Hurlock, L. A. Seals, J.-K. Weng, J. Stout, C. Chapple, REF4 and RFR1, subunits of the transcriptional coregulatory complex Mediator, are required for phenylpropanoid homeostasis in *Arabidopsis*, *J. Biol. Chem*., 287, 5434-5445, 2012.
14. Kim, J. I., W. L. Dolan, N. A. Anderson, C. Chapple, Indole glucosinolate biosynthesis limits phenylpropanoid accumulation in *Arabidopsis thaliana*, *Plant Cell*, 2015.
15. Ito, M., H.J. Okano, R.B. Darnell, R.G. Roeder, The TRAP100 component of the TRAP/Mediator complex is essential in broad transcriptional events and development, *EMBO J*. 21, 3464-3475, 2002.
16. Stevens, J.L., G.T. Cantin, G.Wang, A. Shevchenko, A. Shevchenko, A.J. Berk. Transcription control by E1A and MAP kinase pathway via Sur2 mediator subunit, *Science*, 296, 755–758, 2002.
17. Béve, J., G.-Z. Hu, L.C. Myers, D. Balciunas, O. Werngren, K. Hultenby, R. Wibom, H. Ronne, C.M. Gustafsson, The structural and functional role of Med5 in the yeast Mediator tail module. *J. Biol. Chem*., 280, 41366–41372, 2005.
18. Elmlund, H., V. Baraznenok, M. Lindahl, C.O. Samuelsen, P.J. Koeck, S. Holmberg, H. Hebert, C.M. Gustafsson. The cyclin-dependent kinase 8 module sterically blocks Mediator interactions with RNA polymerase II. *Proc. Natl. Acad. Sci. USA*., 103, 15788-15793, 2006.

19. Knuesel, M.T., K.D. Meyer, C. Bernecky, D.J. Taatjes, The human CDK8 subcomplex is a molecular switch that controls Mediator coactivator function. *Genes Dev*., 23, 439-451, 2009.
20. Lan, W., F. Lu, M. Regner, Y. Zhu, J. Rencoret, S. A. Ralph, U. I. Zakai, K. Morreel, W. Boerjan, J. Ralph, Tricin, a flavonoid monomer in monocot lignification, *Plant Physiol*., 167, 1284-1295, 2015.
21. del Rio, J. C., J. Rencoret, P. Prinsen, Á. T. Martínez, J. Ralph, A. Gutiérrez, Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods, *J. Agric. Food Chem*., 60, 5922-5935, 2012.
22. Lan, W., K. Moreel, F. Lu, J. Rencoret, J.C. del Rio, W. Voorend, W. Vermerris, W. Boerjan, Ralph, J. Maize tricin-oligolignol metabolites and their implications for monocot lignification. *Plant Physiol.* 171, in press 2016.
23. Morreel K., J. Ralph, H. Kim, F. Lu, G. Goeminne, S. Ralph, E. Messens, W. Boerjan. Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem*. Plant Physiol*. 136, 3537-3549, 2004a.
24. Morreel K., J. Ralph, F. Lu, G. Goeminne, R. Busson, P. Herdewijn, J.L. Goeman, J. Van Der Eycken, W. Boerjan, E. Messens. Phenolic profiling of caffeic acid O methyltransferase deficient poplar reveals novel benzodioxane oligolignols. *Plant Physiol*. 136, 4023-4036, 2004b.
25. Morreel, K., Y. Saeys, O. Dima, F. Lu, Y. Van de Peer, R. Vanholme, J. Ralph, B. Vanholme, W. Boerjan. Systematic structural characterization of metabolites in Arabidopsis via candidate substrate-product pair networks. *Plant Cell* 26, 929-945, 2014.
26. Niculaes C., K. Morreel, H. Kim, F. Lu, L.S. McKee, B. Ivens, J. Haustraete, B. Vanholme, R. De Rycke, M. Hertzberg, J. Fromm, V. Bulone, A. Polle, J. Ralph, W. Boerjan. Phenylcoumaran benzylic ether reductase prevents accumulation of compounds formed under oxidative conditions in poplar xylem.*Plant Cell* 26, 3775-3791, 2014.
27. Dima, O., K. Morreel, B. Vanholme, H. Kim, J. Ralph, W. Boerjan, Small glycosylated lignin oligomers are stored in Arabidopsis leaf vacuoles, *Plant Cell*, 27, 695-710, 2015.
28. Yue, F. Lu, S. A. Ralph, J. Ralph, Identification of 4–O–5-units in softwood lignins via definitive lignin models and NMR. *Biomacromolecules*, in press, 2016.
29. Bonawitz, N. D., C. Chapple, Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty?, *Curr. Opin. Biotechnol*., 24, 336-343, 2013.
30. Binns, A. N., R. H. Chen, H. N. Wood, D. G. Lynn, Cell division promoting activity of naturally occurring dehydrodiconiferyl glucosides: Do cell wall components control cell division?, *Proc. Natl. Acad. Sci. U.S.A*., 84, 980-984, 1987.
31. Billett, E. E., H. Smith, Cinnamic acid 4-hydroxylase from gherkin tissues, *Phytochemistry*, 17, 1511-1516, 1978.
32. Chakraborty, M., A. Karun, A. Mitra, Accumulation of phenylpropanoid derivatives in chitosan-induced cell suspension culture of *Cocos nucifera*, *J. Plant Physiol*., 166, 63-71, 2008.
33. Schilmiller, A. L., J. Stout, J.-K. Weng, J. Humphreys, M. O. Ruegger, C. Chapple, Mutations in the *cinnamate 4-hydroxylase* gene impact metabolism, growth, and development in *Arabidopsis thaliana*, *Plant J.*, 60, 771-782, 2009.
34. Yin, Z., W. Wong, W. Ye, N. Li, Biologically active *cis*-cinnamic acid occurs naturally in *Brassica parachinensis*, *Chin. Sci. Bull*., 48, 555-558, 2003.
35. Wong, W. S., D. Guo, X. L. Wang, Z. Q. Yin, B. Xia, N. Li, Study of *cis*-cinnamic acid in *Arabidopsis thaliana*, *Plant Physiol. Bioch*., 43, 929-937, 2005.
36. Moreno-Risueno M.A., J.M. Van Norman, A. Moreno, J. Zhang, S.E. Ahnert, P.N. Benfey. Oscillating gene expression determines competence for periodic Arabidopsis root branching. *Science,* 329, 1306-1311, 2010.
37. Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ, Vernoux T. A novel sensor to map auxin response and distribution at high spatio-temporal resolution. *Nature,* 482, 103-106, 2012.
38. Knobloch, K.H., K. Hahlbrock. 4-Coumarate - Coa ligase from cell-suspension cultures of Petroselinum-hortense-Hoffm - Partial-purification, substrate-specificity, and further properties. *Arch. Biochem. Biophys*., 184, 237-248, 1977.

39. Vanholme R, V. Storme, B. Vanholme, L. Sundin, J.H. Christensen, G. Goeminne, C. Halpin, A. Rohde, K. Morreel, W. Boerjan. A systems biology view of responses to lignin biosynthesis perturbations in Arabidopsis. *Plant Cell*, 24, 3506-3529, 2012.
40. Naseer S, Y. Lee, C. Lapierre, R. Franke, C. Nawrath, N. Geldner. Casparian strip diffusion barrier in Arabidopsis is made of a lignin polymer without suberin. *Proc Natl Acad Sci U.S.A.*, 109, 10101-10106, 2012.
41. Anderson, N. A., Y. Tobimatsu, P. N. Ciesielski, E. Ximenes, J. Ralph, B. S. Donohoe, M. Ladisch, C. Chapple, Manipulation of guaiacyl and syringyl monomer synthesis in *cad-c cad-d* Arabidopsis results in atypical lignin biosynthesis and modified cell wall structure, *Plant Cell* 27, 2195-2209, 2015.
42. Ralph, J., Hydroxycinnamates in lignification, *Phytochem. Rev*., 9, 65-83, 2010.
43. Wilkerson, C. G., S. D. Mansfield, F. Lu, S. Withers, J.-Y. Park, S. D. Karlen, E. Gonzales-Vigil, D. Padmakshan, F. Unda, J. Rencoret, J. Ralph, Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone, *Science*, 344, 90-93, 2014.
44. Sander, J. D., J. K. Joung, CRISPR-Cas systems for editing, regulating, and targeting genomes, *Nat. Biotechnol*., 32, 347-355, 2014.
45. Shan, Q., Y. Wang, J. Li, Y. Zhang, K. Chen, Z. Liang, K. Zhang, J. Liu, J. J. Xi, J.-L. Qiu, C. Gao, Targeted genome modification of crop plants using a CRISPR-Cas system, *Nat. Biotechnol*., 31, 686-688, 2013.
46. Wang, Y., X. Cheng, Q. Shan, Y. Zhang, J. Liu, C. Gao, J.-L. Qiu, Simultaneous editing of three homoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew, *Nat. Biotechnol*., 32, 947-952, 2014.
47. Mir Derikvand, M., J. Berrio Sierra, K. Ruel, B. Pollet, C.-T. Do, J. Thévenin, D. Buffard, L. Jouanin, C. Lapierre, Redirection of the phenylpropanoid pathway to feruloyl malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reducatse 1, *Planta*, 227, 943-956, 2008.
48. Li, Y., J. I. Kim, L. Pysh, C. Chapple, Four isoforms of Arabidopsis 4-coumarate:CoA ligase have overlapping yet distinct roles in phenylpropanoid metabolism, *Plant Physiol*., 169, 2409-2421, 2015.
49. Consortium, T. I. B. G. S., A physical, genetic and functional sequence assembly of the barley genome, *Nature*, 491, 711-716, 2012.
50. Bartlett, J. G., S. C. Alves, M. Smedley, J. W. Snape, W. A. Harwood, High-thoughput *Agrobacterium*-mediated barley transformation, *Plant Methods*, 4, 22, 2008.
51. Yang, Z., C. Steentoft, C. Hauge, L. Hansen, A. L. Thomsen, F. Niola, M. B. Vester-Christensen, M. Frödin, H. Clausen, H. H. Wandall, E. P. Bennett, Fast and sensitive detection of indels induced by precise gene targeting, *Nucleic Acids Res*., 43, e59, 2015.
52. Liu, X., A. Homma, J. Sayadi, S. Yang, J. Ohashi, T. Takumi, Sequence features associated with the cleavage efficiency of CRISPR/Cas9 system, *Sci. Rep*., 6, 19675, 2016.
53. Zadoks, J. C., T. T. Chang, C. F. Konzak, A decimal code for the growth stages of cereals, *Weed Res*., 14, 415-421, 1974.

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