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The phenylpropanoid pathway is responsible for the biosynthesis of a diverse array of metabolites essential for structural integrity, water transport, UV protection, and defense against herbivores and pathogens (Bonawitz and Chapple, 2010). The products of the phenylpropanoid pathway range from complex, insoluble polymers such as lignin and suberin, to soluble flavonoids and hydroxycinnamate esters (HCEs), to volatile compounds used to attract pollinators (Ralph, 2004; Muhlemann et al., 2014). Among these diverse products, by far the most abundant in terms of carbon allocation is the complex heteropolymer lignin, which accounts for a significant portion of the dry weight of not only woody plants, but also of herbaceous plants like Arabidopsis. Lignin is found mainly in xylem, where it is important for resisting the negative pressure generated by transpiration, and in fibers and sclerified parenchyma, where it provides the structural support required for plants to stand upright (Ralph, 2004). In addition to its fundamental role in plant biology, lignin is of interest to humans because decreasing or altering its structure to provide increased cell wall digestibility would greatly increase the cost effectiveness of converting cell wall polysaccharides biofuels (Mottiar et al., 2016). On the other hand, lignin is very energy dense, and it is possible that plants which have been engineered to accumulate higher levels of lignin could provide improved feedstock for direct combustion and electricity generation as part of a diverse bioenergy portfolio (Parsell et al., 2015).

Phenylpropanoid metabolism can be manipulated but not with impunity. Over the last twenty years, a number of experiments, including several from our own laboratory, have greatly expanded our understanding of the phenylpropanoid biosynthetic pathway, such that now at least the catalog of enzymes and biochemical reactions in the pathway appears relatively complete (Bonawitz and Chapple, 2010) although additional players have recently been identified (Vanholme et al., 2013). Particularly important in contributing to this knowledge has been the phenotypic analysis of plants in which phenylpropanoid metabolism is perturbed as a result of mutation or downregulation of genes encoding pathway enzymes. These studies have revealed that phenylpropanoid flux can be largely rerouted in a rational way from one branch of the pathway to another (Mottiar et al., 2016). Whereas the developmental impact of serval of these manipulations of the pathway are relatively mild, defects at a number of other biosynthetic steps lead to a common suite of pleiotropic phenotypes such as dwarfing and sterility, the severity of which is dependent on the strength and location of the metabolic restriction (Bonawitz and Chapple, 2013).

We use sinapoylmalate, a fluorescent soluble phenylpropanoid product found in Arabidopsis and accumulated in leaves as a genetic reporter and molecular marker of pathway activity, and have identified a number of *reduced epidermal fluorescence* (*ref*) mutants that have enhanced our understanding of lignin biosynthesis and the extent to which it can be altered (Fraser and Chapple, 2007). Several of these show no developmental changes whereas others exhibit significant dwarfing and are often sterile. A thorough mechanistic understanding how these developmental phenotypes arise is essential for determining the feasibility of future bioengineering approaches (Bonawitz and Chapple, 2013). Recently, we made a significant breakthrough when we discovered that the extreme dwarfism of the of the *p*-coumaroylshikimate 3'-hydroxylase- (C3′H) deficient *ref8* mutant (Franke et al., 2002) results from gene expression changes that are dependent upon the transcriptional co-regulatory complex Mediator and that may be triggered by the accumulation of atypical phenylpropanoid metabolites (Bonawitz and Chapple, 2010; Bonawitz et al., 2014). Furthermore, recent data suggest that related Mediator-dependent regulatory mechanisms coordinate the transcriptomic and proteomic changes that mediate alterations in carbon allocation to the phenylpropanoid pathway (Bonawitz et al., 2014; Anderson et al., 2015; Kim et al., 2015, see below). Although these regulatory circuits have all been discovered in the context of Arabidopsis mutants, these same mechanisms are presumably responsible for normal regulation of flux into and through the phenylpropanoid pathway in wild-type Arabidopsis and other plant species.

Consistent with the enormous commitment of energy and carbon it requires, the synthesis of lignin is under tight regulatory control. Control of phenylpropanoid metabolism is orchestrated by a complex, hierarchically organized network of transcription factors, many of them MYB and NAC domain-containing proteins. Proteins near the top of the regulatory cascade act to regulate the expression of other transcription factors, while those located at the most downstream points in the network regulate

the transcription of the phenylpropanoid biosynthetic enzymes directly (Taylor-Teeples et al., 2015). At the metabolic/enzymatic level, it has long been known that the initial, and presumed rate-limiting enzyme of the pathway, phenylalanine ammonia-lyase (PAL) is feedback inhibited by its product, cinnamic acid (Lamb, 1979; Bolwell et al., 1986; Mavandad et al., 1990). More recently, a more complex view has been necessitated by the discovery that phenylalanine levels limit lignin deposition in Arabidopsis (Wang et al., 2018) and that post-translational mechanisms are involved in regulating PAL levels in cells (Zhang et al., 2013; Zhang et al., 2015). Two-hybrid analysis identified three Kelch motif repeat containing F-box (KFB) proteins (KFB01, KFB20, and KFB50) that interact with the four PAL isoforms present in Arabidopsis, leading to their ubiquitylation and degradation by the 26S proteasome (Zhang et al., 2013). A later study identified KFB39 as an additional PAL-interacting protein that leads to PAL turnover (Zhang et al., 2015). Overexpression of *KFB39* led to decreased PAL levels and lower accumulation of soluble phenylpropanoids; whereas, RNAi suppression of *KFB39* expression led to their hyperaccumulation, demonstrating the importance of this regulatory node.

The Mediator complex is an important transcriptional co-activator and co-suppressor found in all eukaryotes. In order to influence transcription, gene-specific activators and repressors must interact with basal transcription factors and the rest of the core transcriptional machinery. In eukaryotes, this interaction is bridged by the large, multi-subunit complex known as Mediator, which was first isolated in *S. cerevisiae* as an activity required in order for transcription to proceed *in vitro* (Flanagan et al., 1991). This activity was later attributed to a complex consisting of more than 20 subunits, which was shown to be involved not only in stimulating basal transcription, but also in transmitting information from regulatory *cis*-acting elements to the core promoter (Kim et al., 1994). Mediator can be divided into three domains, a head domain and a middle domain that interact with RNA polymerase II, and a tail domain more distal to the basal transcription machinery as well as an inhibitory kinase module. Subsequent biochemical and genetic analysis of Mediator in different species has revealed that many Mediator subunits are conserved among the eukaryotes, particularly those that are thought to form contacts with RNA polymerase and the basal transcription machinery (Bourbon et al., 2004; Dolan and Chapple, 2017). In addition to these core subunits, there are a number of other more highly variable Mediator components that are responsible for the interaction of Mediator with process- or taxa-specific transcriptional activators and suppressors (Bäckström et al., 2007, Borggrefe and Yue, 2011). For example, the yeast co-repressor dTMP-Uptake 1 (Tup1) and its *Arabidopsis* ortholog LEUNIG (LUG) both interact with MED14 to repress transcription (Conlan et al., 1999; Gonzalez et al., 2014). In yeast, Tup1-mediated repression also requires CDK8 at some loci (Green and Johnson, 2004). Likewise, in *Arabidopsis,* LUG has been shown to directly interact with CDK8, and in yeast strains in which CDK8 or MED14 were deleted, repression activity of a LUG-LexA fusion was reduced (Gonzalez et al., 2014). Together, these results suggest that the orthologous co-repressors LUG and Tup1 function through conserved interactions with orthologous MED subunits. Another *Arabidopsis* Mediator-transcription factor interaction that was identified based on an orthologous interaction in yeast is that of MED18 and the zinc finger transcription factor YIN YANG 1 (YY1). Based on the knowledge that yeast MED8 interacts with the transcription factor Ace2, and that MED8 and MED18 have overlapping roles in yeast, Lai et al. reasoned that *Arabidopsis* MED8 or MED18 might interact with an ortholog of Ace2 (Lai et al., 2014). They identified the uncharacterized protein YY1 as the closest *Arabidopsis* ortholog of Ace2 and found that it interacted with MED18, but not MED8, in both bimolecular fluorescence complementation and coimmunoprecipitation assays. MED25 was first identified as PHYTOCHROME AND FLOWERING TIME 1 (PFT1) for its role in promoting flowering under far-red light (Cerdán and Chory, 2003) and is another hub for the transcriptional regulation of abiotic and biotic stress responses. It is also a common target of transcription factors, with more than 20 interactions identified to date (reviewed in Yang et al., 2015). Since then, it has been shown to function in numerous stress and growth-related pathways, in some cases as a positive regulator and in others as a negative regulator.

Despite the clear importance of Mediator throughout the eukaryotes, there are as yet only a modest number of published studies that deal with the function and composition of plant Mediator. Mediator has been implicated in the control of a number of biological processes in plants, such as

flowering (Cerdán and Chory, 2003; Bäckström et al., 2007), pathogen defense (Dhawan et al., 2009; Kidd et al., 2009), pectin and cellulose biosynthesis (Sorek et al., 2015), cold tolerance (Boyce et al., 2003; Bäckström et al., 2007), and iron homeostasis (Yang et al., 2014) but until recently there had not been any suggestion that Mediator plays a role in the regulation of phenylpropanoid biosynthesis. Experiments aimed at determining the subunit composition of Arabidopsis Mediator employed two different antibodies to immunoprecipitate the complex and identified co-precipitating proteins by mass spectrometry (Bäckström et al., 2007). Among the likely Arabidopsis Mediator subunits were two related proteins which were designated MED33a (At3g23590) and MED33b (At2g48110). Although At3g23590/MED33a and At2g48110/MED33b were originally suggested to be plant-specific Mediator components, more recent phylogenetic evidence has suggested that both are, in fact, orthologous to yeast MED5 and mammalian MED24 (Bourbon, 2008).

We studied the function of Mediator in the regulation of metabolism in Arabidopsis in four distinct but highly complementary contexts as described below.

1. MED5a and MED5b are required for normal regulation of phenylpropanoid pathway product accumulation. An allelic series of three mutants, *ref4-1*, *ref4-2*, and *ref4-3*, exhibit global decreases in phenylpropanoids, dwarfing, and dark-green spatulate leaves (Ruegger and Chapple, 2001). These mutations, all of which exhibit semi-dominant inheritance, map to At2g48110, leading to D647N (*ref4-1* and *ref4-2*) and G383S (*ref4-3*) substitutions in the REF4/MED33b/MED5b protein (Stout et al., 2008). Plants homozygous for *REF4* null alleles are morphologically and metabolically indistinguishable from wild-type plants, presumably due to the existence of the *REF4* paralog *REF FOUR RELATED1* (*MED33A/MED5a*). Neither *med5a med5b* nor *med5* single null plants morphologically resemble *ref4-3* plants, as they develop normal rosette leaves and attain wild-type height at maturity (Bonawitz et al., 2012). In contrast, although they develop relatively normally, both *med5a* and *med5a med5b* plants hyperaccumulate phenylpropanoids in multiple tissues (Bonawitz et al., 2012). These data show that MED5a and MED5b are required for phenylpropanoid homeostasis in wild-type plants, and strongly suggest that the phenotypes of the original *ref4-1*, *ref4-2*, and *ref4-3* mutants are due to enhanced activity of the mutant *med5a* gene products, which directly or indirectly leads to constitutive repression of phenylpropanoid metabolism.

2. Restoration of growth of a lignin-deficient mutant by elimination of MED5 function implicates Mediator in a metabolic feedback process. We generated *med5a med5b ref8-1* triple mutants and made the serendipitous discovery that this led to almost complete rescue of the severe *ref8-1* growth phenotype, with the morphology and fertility of *med5a med5b ref8-1* plants being virtually indistinguishable from that of *med5a med5b* plants (Bonawitz et al., 2014). Further, although *ref8-1* mutants contain only 40% of the amount of lignin found in wild-type plants, lignin deposition in *med5a med5b ref8-1* triple mutants is restored to wild-type levels (Bonawitz et al., 2014). On the basis of these results, we concluded that MED5a and MED5b, and by extension Mediator, play an active role in generating both the lignin deficiency and the dwarf phenotype of Arabidopsis *ref8* mutants. To determine the possible effects on gene expression of disrupting *MED5a* and *MED5b* in the *ref8-1* mutant background, we performed RNAseq to identify differentially expressed genes among wild-type, *med5a med5b*, *ref8-1*, and *med5a med5b ref8-1* plants. Consistent with our previous report (Bonawitz et al., 2012), *med5a med5b* plants exhibited elevated transcript levels of phenylpropanoid biosynthetic genes, including those encoding PAL, C4H, 4CL, C3´H, CCR, CAD, and CSE. Moreover, “phenylpropanoid biosynthesis” was the term most significantly over-represented among gene ontologies of the 248 genes overexpressed in *med5a med5b* plants (Bonawitz et al., 2014). In *ref8-1* plants, a total of 8772 genes, representing a substantial fraction of the genome, were expressed at levels significantly different from wild type. Remarkably, of these genes, over 90% were expressed at wild-type levels, or were are misregulated to a lesser degree, in *med5a med5b ref8-1* plants. Taken together, these results demonstrate that the metabolic disruption in *ref8-1* plants results in widespread qualitative and quantitative changes in gene expression in *ref8-1* mutant plants, and are consistent with a model in which MED5a and MED5b are required, directly or indirectly, for sensing the metabolites that hyper-accumulate in the absence of normal levels of C3´H activity.

3. The accumulation of indole-3-acetaldoxime or an aldoxime derivative perturbs phenylpropanoid biosynthesis in a Mediator-dependent fashion. The *ref5-1* mutant is sinapoylmalate-deficient but is not defective in an enzyme involved in phenylpropanoid biosynthesis. Instead, it is defective in an enzyme of glucosinolate metabolism, the cytochrome P450-dependent monooxygenase CYP83B1, indicating that there is metabolic cross-talk between these two pathways (Bak et al., 2001; Ruegger and Chapple, 2001; Hansen et al., 2001; Kim et al., 2015). Its paralog, CYP83A1, is defective in the *ref2* mutant and its disruption leads to similar phenylpropanoid phenotypes (Hemm et al., 2003). The enzymes CYP79B2 and CYP79B3 generate the substrate for CYP83B1, indole acetaldoxime (IAOx), from tryptophan (Sugawara et al., 2009; Zhao et al., 2002). Importantly, mutations in these enzymes generate the opposite phenotype seen in *ref2* and *ref5*: sinapoylmalate levels are increased in *cyp79b2 cyp79b3* and in the *ref5-1 cyp79b2 cyp79b3* triple mutant (Kim et al., 2015). These results indicate that a) the perturbation of phenylpropanoids in *ref5* mutants is not due to a deficiency in indole glucosinolates, b) the crosstalk between the glucosinolate and phenylpropanoid pathways is relevant in wild-type plants and c) cellular levels of the *REF5*/CYP83B1 substrate IAOx (or a derivative) are sensed by some mechanism and impact the level of phenylpropanoids accumulated.

Upon further examination, we found that in addition to sinapoylmalate, coniferin, syringin, and flavonoids were reduced in *ref5* and enhanced in *cyp79b2 cyp79b3* mutants. These results suggested that the cause of phenylpropanoid perturbation in *ref5* mutants is in a common step early in the pathway (Kim et al., 2015). To test this hypothesis, we measured PAL activity in seedling extracts and found that *ref5-1* and *ref5-2* mutants (a null T-DNA insert) contain about 70% and 25% of wild-type PAL activity, respectively. To identify genes required for the crosstalk between glucosinolate biosynthesis and phenylpropanoid metabolism, we performed a *ref5* suppressor screen and isolated three independent lines that had restored sinapoylmalate fluorescence under UV light. Whole genome sequencing analysis revealed that all three of the suppressors have mutations in the same gene, At3g23590, which encodes MED5a. The observation that perturbation of a major transcriptional co-regulatory complex restores phenylpropanoid metabolism in the *ref5* mutant background strongly suggests that the crosstalk between the glucosinolate and phenylpropanoid biosynthetic pathways occurs at the transcriptional level.

4. Anthocyanin accumulation is repressed in a Mediator-dependent manner in *fah1* mutants. **S**oil-grown *fah1* plants and sucrose-stressed seedlings exhibit a clear reduction in visible purple pigmentation attributable to anthocyanin accumulation. The cause of this phenotype was unclear given that F5H is not required for the synthesis of the phenylpropanoid-derived anthocyanin nucleus (Maruta et al., 2014; Anderson et al., 2015). Although F5H is required for the formation of the sinapate moiety that decorates the most abundant anthocyanin in Arabidopsis leaves (A11 according to the nomenclature of Tohge et al., 2005), the addition of this acyl group is not required for anthocyanin accumulation (Fraser et al., 2007). The Arabidopsis mutant *pap1-D* is an activation tagged line that hyperaccumulates anthocyanins due to constitutive expression of the R2-R3 MYB transcription factor MYB75 (Borevitz et al., 2000), which stimulates transcription of multiple genes in the anthocyanin biosynthetic pathway including those encoding PAL. To test whether overexpression of *PAP1* can restore anthocyanin biosynthesis in plants lacking *F5H*, we generated *fah1 pap1-D* double mutants. Initial visual inspection and subsequent HPLC analysis of *fah1 pap1-D* plants revealed that tissues of *fah1 pap1-D* plants contained significantly lower levels of total anthocyanins than leaves of *pap1-D* plants, but only in tissues where *F5H* is normally expressed (Anderson et al., 2015). Surprisingly, analysis of *med5a med5b fah1* and *med5a med5b fah1 pap1-D* plants showed that anthocyanin accumulation is restored in this genetic background. These data indicate that the inhibition of anthocyanin accumulation in *fah1*, is Mediator-dependent. Given that *ref8 fah1* double mutants accumulate normal levels of anthocyanins (Anderson et al., 2015), our working hypothesis is that a metabolite (or derivative thereof) downstream of C3'H but upstream of F5H is sensed directly or indirectly by MED5, leading to transcriptional changes that suppress anthocyanin accumulation.

Based on the data described above, we concluded that MED5a and MED5b, acting as components of the Mediator complex negatively influence the accumulation of phenylpropanoid metabolites (*fah1*, *ref2*, *ref4*, *ref5*, *ref8*) and plant growth (*ref4*, *ref8*) by participating in transcriptional co-activation and/or

co-suppression of critical target genes. We postulated that metabolites (from within the phenylpropanoid pathway or intermediates in glucosinolate biosynthesis) accumulate at aberrant levels and are recognized by a yet-to-be-identified system that then leads to MED5-dependent changes in gene expression. To gain more insight into this novel aspect of phenylpropanoid metabolic regulation and how the growth perturbations seen in lignin-modified plants come about, over the last funding period we 1) explored the mechanism(s) involved in phenylpropanoid suppression in Arabidopsis mutants, 2) characterized *ref4-3* suppressor mutants, and 3) identified additional genes involved in phenylpropanoid homeostasis. This research has resulted in the six manuscripts below, two of which are in preparation or in the review process.

Our first manuscript focused on the identification and characterization of suppressors of the *ref4-3* mutant of Arabidopsis (Dolan WL, Dilkes BP, Stout JM, Bonawitz ND, Chapple C (2017) Mediator Complex Subunits MED2, MED5, MED16, and MED23 genetically interact in the regulation of phenylpropanoid biosynthesis. Plant Cell **29**: 3269-3285). The mutant protein encoded by the *ref4-3* allele of *MED5b* constitutively downregulates phenylpropanoid accumulation and leads to dwarfing. To identify genes required for *ref4-3* function, or for the phenotypes that the mutant exhibits, we performed a genetic screen for mutants that suppress *ref4-3*. The molecular identities of these suppressor alleles were determined by whole-genome sequencing and confirmed by complementation tests with additional alleles. We showed in this manuscript that all but one of the suppressors of the *ref4-3* allele of *MED5b* that we isolated harbor intragenic mutations in *MED5b* or mutations in the *MED2*, *MED16*, and *MED23* subunits of Mediator. Transcriptomic analysis of the mutants suggests that *ref4-3* represses phenylpropanoid biosynthesis by upregulating negative regulators of the pathway. As mentioned previously, *KFB01*, *KFB20*, *KFB39* and *KFB50* encode F-box proteins that regulate the ubiquitination and subsequent degradation of PAL (Zhang et al., 2013, 2015). *KFB01*, *KFB39*, and *KFB50* were upregulated in *ref4-3*, with *KFB39* showing the greatest increase (13-fold) in expression. In the suppressors, *KFB39* upregulation was diminished to an extent consistent with the restoration of sinapoylmalate accumulation in each line ($r = -0.72$). *KFB39* was also downregulated in *med5ab* more than 4-fold, which likely contributes to the increased content of soluble phenylpropanoids in that mutant background. Together, these data suggest that proteasome-mediated turnover of PAL contributes to the reduced phenylpropanoid content of *ref4-3*. In addition, two of the suppressors we identified restore growth without restoring soluble phenylpropanoid accumulation, demonstrating that reduced phenylpropanoid accumulation is not likely to be the proximal cause of growth inhibition in the mutant.

Our second manuscript examined our RNAseq data in more detail to provide a better understanding of the role of each MED tail subunit in gene expression (Dolan WL, Chapple C. (2018) Transcriptome Analysis of Four Arabidopsis thaliana Mediator Tail Mutants Reveals Overlapping and Unique Functions in Gene Regulation. G3 **8**: 3093-3108). In this work, we explored the functions of four Arabidopsis Mediator tail subunits, MED2, MED5a/b, MED16, and MED23, by comparing the impact of mutations in each on the Arabidopsis transcriptome. We found that these subunits affect both unique and overlapping sets of genes, providing insight into the functional and structural relationships between them. We found evidence for a tissue specific role for MED23, as well as in the production of alternatively spliced transcripts. Together, these data help disentangle the individual contributions of these MED subunits to global gene expression and suggest new avenues for future research into their functions.

We were also invited to write three review articles that brought together our work on Mediator and phenylpropanoid metabolism and also explored the link between phenylpropanoid pathway modification and the dwarfing that is often associated with lignin-modified plants (Dolan WL, Chapple C (2017) Conservation and divergence of Mediator structure and function: insights from plants. Plant Cell Physiol **58:** 4-21; Muro-Villanueva F, Mao X, Chapple C (2019) Linking phenylpropanoid metabolism, lignin deposition, and plant growth inhibition. Curr Opin Biotech, 56: 202-208; Mao X, Weake VM, Chapple C (2019) Mediator function in plant metabolism revealed by large-scale biology. J. Exp Bot, 70: 5995-6003).

We have also conducted a detailed analysis of the *cdk8* mutant because we identified it independently as a suppressor of the *ref4-3* mutant (Mao X, Kim JI, Wheeler MT, Heintzelman AK,

Weake VM, Chapple C (2019) Mutation of Mediator subunit CDK8 counteracts the stunted growth and salicylic acid hyper-accumulation phenotypes of an Arabidopsis MED5 mutant. New Phytol **223**: 233-245). We focused on CDK8 because the kinase module has been shown to interact with other Mediator subunits in yeast and humans where it is known to act as a repressive component (Elmlund et al., 2006; Knuesel et al., 2009). In *ref4-3 cdk8* the growth defect phenotypes of *ref4-3* are largely rescued but the content of soluble metabolites and total lignin deposition are as low as those in *ref4-3*, and this rescue is specifically dependent upon loss of the kinase activity of CDK8. RNAseq analysis revealed that among the genes that showed greatest mis-regulation in *ref4-3*, *DJC66*, a gene encoding a small J-domain containing protein, was up-regulated more than 23-fold in *ref4-3* compared to wild type, and its expression was partially rescued in *ref4-3 med2*, *ref4-3 med16*, *ref4-3 med23*, and *ref4-3 cdk8*. Based upon these characteristics, we pursued this gene as one that could be involved in dwarfing. Loss of DJC66 partially suppresses the growth deficiency of *ref4-3*, but as with suppression by *cdk8*, the phenylpropanoid accumulation in *ref4-3 djc66* was as low as that in *ref4-3*. The identification of DJC66 as a weak suppressor of *ref4-3* suggests that transcriptome analysis can be a feasible approach to identify the genes that are responsible for the Mediator-dependent phenotypes exhibited in phenylpropanoid mutants. Further, these findings provide additional evidence that the growth and metabolic phenotypes of at least some phenylpropanoid mutants can be disentangled from one another and suggest that dwarfing in *ref4-3* plants may primarily be the result of aberrant gene expression as it is in *ref8* (Bonawitz et al., 2014), rather than the direct result of a perturbation of lignin deposition.

Next, we planned to identify additional genes required for phenylpropanoid homeostasis by testing two other hypotheses: *1) loss of multiple tail subunits will phenocopy the ref8 growth suppression exhibited in ref8 med5a med5b, and 2) suppressors of the fah1 anthocyanin-deficiency phenotypes will include Mediator subunit genes and those encoding additional proteins involved in feedback regulation of phenylpropanoid metabolism.* To test hypothesis 1, we crossed *ref8* mutants with a set of other *MED* knockout mutants, starting with those that encode Mediator subunits that are *ref4-3* suppressors, reasoning that mutants that genetically interact with the dominant *MED5b* allele are most likely to share with the *med5a med5b* double knockout a common ability to suppress *ref8* phenotypes. Measurements of canopy area quantified using ImageJ and measurements of stem height revealed that *med2 ref8*, *med16 ref8* and *med23 ref8* double mutants were largely indistinguishable from *ref8* mutants. In contrast, of the three higher order mutants generated from these double mutants, *med16 med23 ref8* exhibited substantial growth rescue and restored fertility, although rescue of plant height was less than seen in *med5a med5b ref8*. These results implicate the function of additional Mediator subunits in the growth perturbation seen in *ref8*. The observation that the phenotype of *med16 med23 ref8* is not as strong as *med5a med5b ref8* further suggests that the absence of MED16 and MED23 do not suppress the phenotype of *ref8* simply by eliminating MED5 from the complex.

To test hypothesis 2, we reasoned that since the anthocyanin-deficiency phenotype of *fah1* is exhibited robustly in *fah1 pap1-d*, this double mutant background would be ideal starting material for a mutant screen to identify other genes/proteins involved in this feedback process. We thus performed an ethyl methanesulfonate (EMS) mutagenesis and screened for mutants with restored anthocyanin biosynthesis. Fourteen M1 families were collected and approximately 600,000 M2 plants were screened visually for overproduction of the anthocyanin pigment (more like *pap1-D*), compared to *fah1 pap1-D* controls. We identified over 100 putative suppressors from this initial screen. Their individual progeny were then re-screened for higher anthocyanins by HPLC (n=4). In the end, 25 lines exhibited a statistically significant increase ($\alpha < 0.05$) in anthocyanins relative to *fah1 pap1-D* ranging from 1.5-4.0-fold higher than *fah1 pap1-D*. To identify candidate genes that contributed to the suppression, we used whole-genome sequencing to detect intragenic single nucleotide polymorphisms as we had done to identify *ref4-3* suppressors (Dolan et al., 2017). Five of the suppressors that had the highest accumulation of anthocyanins, all from independent M1 families, contained mutations in the RNA-silencing complex, including RNA-DEPENDENT RNA POLYMERASE 1 (RDR-1; 3 different alleles) and SUPPRESSOR OF GENE SILENCING1 (SGS-1; 2 different alleles) (Xie & Qi, 2008). Two weaker suppressors contain independent mutations in a gene annotated as an RNA recognition motif protein of unknown function.

This preliminary result suggests that lack of F5H expression may induce silencing of anthocyanin biosynthetic genes through an RNA-dependent gene silencing mechanism as has been reported for wax biosynthesis in Arabidopsis (Lam et al., 2012; Lam et al., 2015). Of note, although we know that *MED5* is required for anthocyanin suppression in *fah1*, we have yet to find mutations in it or other Mediator complex genes, possibly due to genetic redundancy between *MED5a* and *MED5b*. At this time we do not know whether the RDR-1/SGS-1 functions upstream or downstream of Mediator in this metabolite accumulation suppression phenomenon.

In order to gain mechanistic insights into Mediator function in the future, we determined it was essential to develop methods to immunoprecipitate (IP) Mediator from plant extracts and determine its subunit composition, understanding that these results would represent a tissue-average if any subunits have a tissue-specific distribution. To accomplish this goal, we have epitope-tagged a number of Mediator subunits and have had success with FLAG-tagged MED21. We chose this subunit because *med21* mutants display an embryo lethal phenotype (Dhawan et al., 2009) that would provide a critical test for the functional incorporation of the epitope-modified subunit into the Mediator complex. Our MED21 C-terminal FLAG construct driven by the native *MED21* promoter complements the embryo-lethal phenotype of *med21* and leads to plants that develop normally. Using this line, we prepared extracts of *med21 MED21-FLAG* seedlings, IP'd using anti-FLAG beads, eluted with FLAG peptide and analyzed the eluate by mass spectrometry (MS) at the Purdue Proteomics Facility. This analysis identified 31 Mediator subunits, many of which had good peptide coverage and high Mascot scores. Importantly, we were able to identify all Mediator tail subunits including MED2, MED5a, MED5b, MED16, and MED23, all of which are critical to the regulation of phenylpropanoid metabolism and the dwarfing seen in *ref8* and/or *ref4-3*. It is also important to note that we were able to identify peptides from five kinase module subunits, even though the kinase module has not been co-purified with plant Mediator in the past (Bäckström et al., 2007), suggesting that our method can preserve even weakly interacting partners within the complex. We have also had good success with chromatin immunoprecipitation (ChIP) using a line expressing FLAG-tagged MED16 in a *med16* background (Wang et al., 2015).

To elucidate the molecular mechanisms by which Mediator affects gene expression, we optimized a previously published ChIPseq protocol (Saleh et al., 2008), and used this to first determine the genome-wide distribution of Pol II in wild-type, *ref4-3*, *ref4-3 cdk8*, and *med5* plants, and compared these data with our RNAseq analysis of the same mutants. We found that Pol II occupancy at all annotated genes was positively correlated with their expression level in wild-type plants, and identified regions of differential Pol II occupancy in the *med5* mutants compared to wild type. Most of these displayed increased Pol II occupancy in *ref4-3* and decreased Pol II occupancy in *med5*. Further, we identified gene targets that were both differentially expressed, and showed altered Pol II occupancy, in *ref4-3* in a CDK8-dependent manner, including *KFB39* and *KFB50* and the previously mentioned DNA J protein. In summary, our data supports a model in which MED5 activates negative regulators of phenylpropanoid metabolism and plant growth and in which the mutant form of the protein encoded by *ref4-3* enhances this capacity or removes regulatory constraints on it. Having optimized Pol II ChIP, we then backcrossed the line expressing a FLAG-tagged version of the tail module subunit MED16 into different *med* mutant backgrounds, and generated chromatin preparations for Mediator ChIP-qPCR with strong signal and low background results.

MODEL

The molecular events underlying the homeostatic mechanisms governing phenylpropanoid metabolism and the alterations of growth seen in lignin-modified plants are complex and poorly understood. We have shown that disruption of both *MED5a* and *MED5b* results in enhanced expression of multiple phenylpropanoid biosynthetic genes and down-regulation of proteins involved in post-translational regulation of the pathway, with a concomitant increased accumulation of numerous downstream products. The dominant *MED5b* allele in the *ref4-3* mutant has the opposite effect and simultaneously leads to growth defects. We have also shown that elimination of the Mediator subunits MED5a and MED5b rescues the stunted growth, lignin deficiency, and widespread changes in gene

expression exhibited by the phenylpropanoid pathway mutant *ref8*, without restoring the synthesis of guaiacyl and syringyl lignin subunits. We have found that MED5b is required for the crosstalk between the glucosinolate and phenylpropanoid pathways, and that both MED5 paralogs are involved in the inhibition of anthocyanin accumulation in *fah1*. Taken together with the established roles of Mediator in transcriptional regulation, and of MED5a and MED5b in the repression of phenylpropanoid metabolism, these observations lead us to propose the following model. MED5a and MED5b are key components of an active, transcriptional process by which phenylpropanoid homeostasis is maintained in wild-type plants. In plants in which phenylpropanoid or glucosinolate metabolism is perturbed (*ref8*, *fah1*, *ref2* and *ref5* mutants), the metabolic blocks lead to changes in one or more soluble metabolites that is sensed directly or indirectly by/through MED5. In *ref8* plants, this elicits an inappropriate or exaggerated response by this normally homeostatic pathway and initiates a transcriptional cascade that ultimately results in repression of lignification and impaired growth. In *ref2* and *ref5* plants, the level of IAOx or its derivative impacts PAL activity and possibly other enzyme activities via F-box mediated PAL ubiquitylation and degradation. The phenylpropanoid phenotypes of the *ref4-3* mutant also appear to come about via F-box gene upregulation and the impacts on plant growth require mis-regulation of a DNA J protein. Finally, the down-regulation of anthocyanin accumulation in *fah1* involves MED5 as well, but our recent evidence also suggests the involvement of additional cellular machinery involved in RNA-mediated gene silencing.

SIGNIFICANCE

Understanding the molecular mechanisms by which carbon flux is partitioned in plants, and how flux through the phenylpropanoid pathway in particular is regulated and allocated to different branches of the pathway is essential for the eventual rational manipulation of phenylpropanoid flux for bioenergy applications. In this proposal, we outline experiments aimed at expanding our understanding of the role of Mediator, as well as elucidating the mechanisms by which these proteins influence phenylpropanoid biosynthesis and growth and how metabolite accumulation within the pathway and between metabolic pathways is essential for feedback regulatory control. These experiments will inform future bioengineering efforts aimed at altering carbon allocation and cell wall engineering by contributing to our understanding of the role of Mediator in regulating lignin biosynthesis, a pathway of plant metabolism that is important on a global scale.

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