ABSTRACT

PANDA, CHINMAYEE. Isolation and characterization of a suppressor of an Arabidopsis lignin-deficient dwarf mutant. (Under the direction of Dr. Xu "Sirius" Li).

Currently little is known about the genetic and cellular mechanisms of Lignin Modification Induced Dwarfism. Identification and characterization of genetic suppressors of Arabidopsis lignin deficient mutants can be a powerful approach in understanding the molecular mechanism(s) mediating growth defects in low lignin mutants with high saccharification efficiency. In this study, we performed a suppressor screen with the Arabidopsis *C3'H* mutant, *ref8* (*reduced epidermal fluorescence 8*) to identify new genes involved in the lignin modification induced dwarfism (LMID) pathway. Defects in the *C3'H gene* result in low lignin plants with reduced sinapate esters accumulation, dwarf stature, collapsed xylem and female sterility. Suppressor lines exhibiting significant growth restoration were backcrossed to generate mapping populations. Loci for Growth Inhibition Relieved genes (*GIR*), were mapped using bulked segregant sequencing, and functional complementation and double mutant analyses were carried out to confirm their involvement in LMID.

Two suppressors were further characterized in detail at the biochemical, genetic and cellular levels to investigate the molecular mechanisms involved in growth rescue. While GIR1 is annotated as a beta-importin, GIR2 is a known transcription factor. In the first suppressor, *ref8 gir1,* lignin content or composition was only partially restored with collapsed xylem vessels still persistent. The soluble metabolite phenotype of both *ref8 gir1* and *ref8 gir2* plants was not restored suggesting that *C3'H* is still blocked. As part of the study, we discovered that *ref8* is defective in both cell proliferation and expansion and these cellular defects are significantly rescued in both suppressor lines. We generated *ref8 gir1*

gir2 triple mutant to understand if these two *GIR* genes interact genetically. The *ref8 gir1 gir2* triple mutants grow much bigger than either *ref8 gir1* or *ref8 gir2* plants, suggesting that *gir1* and *gir2* have an additive effect on *ref8* growth and are likely to be on independent pathways. Our data revealed that *gir1* cannot rescue other lignin biosynthesis mutants like *ccr1* and *ref3-2*, suggesting that dwarfism in these mutants may be due to different mechanisms. Interestingly, *gir1* was able to rescue *ref4-3*, a dominant gain of function mutant of REF4, which is a subunit of a large multiprotein transcription modulator called Mediator. Rescue of *ref4-3* dwarfism by *gir1* suggests a common cause for dwarfism in both *ref8* and *ref4-3* mutants. Identification of interacting partners of GIR1 followed by identification and characterization of downstream targets of MYB4 and GIR2 in *ref8* background, will be instrumental in elucidating the genes and pathways that mediate LMID.

Isolation and Characterization of a Suppressor of an Arabidopsis Lignin-deficient Dwarf Mutant.

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Biology

Raleigh, North Carolina

2017

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DEDICATION

To Lord Ganesha and Maa Saraswati

To my parents, Dillip Panda and Basanti Kar

To my brother, Abhilash Panda

And most of all, to my husband and best friend, Anand Nanda who motivated me to pursue this journey and whose love, patience and prayers allowed for its completion.

BIOGRAPHY

Chinmayee Panda was born in Balasore, a coastal city in the state of Orissa, India. She is the older child of Dillip Panda and Basanti Kar. She went to St. Vincent's convent school, which imparts schooling until higher secondary level. In 2002, she joined F.M autonomous college where she finished her 11th and 12th board studying Physical Sciences with a major in Biology and Mathematics. In 2004, she joined Amity Institute of Biotechnology, Noida, India, where she earned a bachelor's degree in technology in 2008. She immersed herself in corporate culture for 3 years obtaining training in the biotech industry. Chinmayee moved to US in 2011 with her husband and enrolled in the Plant Biology graduate program at North Carolina State University in the fall of 2012 under the supervision of Dr. Xu "Sirius" Li. She won several awards during her graduate career at NC State, including a second place in NCSU graduate research symposium, 2016 and a first place in the NCRC poster competition, 2015.

ACKNOWLEDGEMENTS

I would like to offer my sincere gratitude to Dr. Xu "Sirius" Li for taking on the role and responsibility for being my advisor. His drive for excellence in research inspired me to try to be a better researcher. I greatly appreciate his advice and support. He has been very patient and encouraging throughout the training. I would not have completed my dissertation without his guidance. I sincerely thank my committee members for their valuable guidance and continuous encouragement. My deepest thanks goes to my professors for great lectures in Plant Biology and making this academic journey truly memorable. A special thanks to Dr. Renee' Strauch for her assistance and advice during my initial years in the Li lab. My sincere thanks to Dr. Han-Yi-Chen, Dr. Amanda Wagner and Samantha Case in the Li Lab for their tremendous support, encouragement, valuable feedback and fun times. I would like to thank Dr. Tzung Fu Hsieh, Dr. Changqing Zhang and Yu-Hung Hung for being great lab neighbors and providing valuable feedback. I would like to thank my parents, Dillip Panda and Basanti Kar, my brother Abhilash Panda for their moral support and encouragement. My deepest gratitude goes to my wonderful husband, Anand, for his love, patience, and encouragement during my PhD studies. Lastly, I would like to thank God for showing me a ray of hope during difficult times.

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LIST OF ABBREVIATIONS

LMID: lignin modification induced dwarfism

REF: reduced epidermal fluorescence

GIR: growth inhibition relieved

PAL: Phenylalanine ammonia lyase

C4H: Cinnamate 4-hydroxylase

4CL: 4-Coumarate: CoA Ligase

C3H: *p*-Coumarate 3-hydroxylase

CSE: Caffeoyl shikimate esterase

HCT: *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyltransferase

CCoAOMT: Caffeoyl-CoA O-methyltransferase

CCR: Cinnamoyl-CoA reductase

F5H: Ferulate 5-hydroxylase

COMT: Caffeic acid O-methyltransferase

CAD: Cinnamyl alcohol dehydrogenase

SAD2: Supersensitive to abscisic acid and drought 2

CHS: Chalcone synthase

PR: Pathogenesis related

DAP: Days after planting

EMS: Ethyl methanesulfonate

CHAPTER 1

Introduction to Lignin Modification Induced Dwarfism (LMID)

The limitations of first generation biofuels in achieving sustainable targets for climate change mitigation and economic growth has increased the interest in the production and improvement of second generation or advanced biofuels (Antizar- Ladislao and Turrion-Gomez, 2008). While first generation biofuels are mainly obtained from sugars and oilseeds of traditional food crops like sugarcane, corn, beet root, and wheat, current second generation biofuels are made from woody and fibrous biomass obtained from non-food parts of bioenergy feed stocks like switchgrass, poplar, alfalfa, jatropha and maize (Havlík, et al., 2011; Naik, et al., 2010; de Vries, et al., 2010). Bioethanol production from agricultural residues like corn stover and bagasse, short rotation woody crops, forestry residues and paper waste are also considered as attractive alternatives (Kim and Dale, 2004; Kadam and McMillan, 2003).

Lignocellulosic biomass composed of cellulose, hemicelluloses and lignin is considered a potential bioenergy feedstock that is both sustainable and economically viable (Hamelinck, et al., 2005). However, compared to the easy processing of bioethanol from first-generation biofuel feedstocks, presence of lignin limits the use of lignocellulose due to “cell wall recalcitrance” by extreme resistance to enzymatic digestibility (Hendriks and Zeeman, 2009; Himmel, et al., 2007a). Lignin, a phenolic polymer deposited in the secondary cell walls of plant vascular tissues, forms a rigid physical barrier preventing access to sugars embedded within cell walls for biofuel production, also known as the “recalcitrance problem”(Himmel, et al., 2007a).

Biologically, lignin is a very important structural polymer that confers mechanical strength, rigidity and hydrophobicity to plant cell walls (Boudet, et al., 2003; Humphreys and Chapple, 2002b). The structural and chemical properties of lignin, helps plants withstand negative pressure of transpiration and thereby, conduct water efficiently (Wang, et al., 2013; Yoon, et al., 2015). For efficient utilization of plant biomass for agro-industrial applications, total lignin content must be lowered or amount of easily degradable lignin increased by genetically modifying enzymes involved in lignin biosynthesis (Baxter and Stewart Jr, 2013; Himmel, et al., 2007b; Liu, et al., 2014).

While many reports suggest a high correlation between reduced lignin content and glucose yield resulting from genetic engineering of lignin pathway, a major limitation to this strategy includes severe morphological defects and subsequently reduced biomass in some lignin modified plants (Coleman, et al., 2008b; Chapple, et al., 1992; Li, et al., 2008a; Baxter and Stewart Jr, 2013). The growth defects include dwarf stature, reduced fertility, vascular collapse and increased susceptibility to pathogen infection, collectively referred to as LMID (Bonawitz and Chapple, 2010; Bonawitz and Chapple, 2013; Coleman, et al., 2008a).

Little is known about the molecular mechanisms underlying LMID (Bonawitz and Chapple, 2013). Isolation of suppressors of low lignin dwarf mutants is a powerful strategy to identify genes that underpin LMID. Characterization of these suppressors can shed light on the complex mechanism by which LMID is mediated upon phenylpropanoid pathway perturbation.

1.1 Evolutionary aspects of lignification: Biosynthesis, structure and function

Many years of investigation have indicated that the biosynthesis of lignin evolved along with colonization of land by plants during the late Ordovician, around 450 million years ago (Stewart and Rothwell, 1993; Robinson, 1990). Early theories on evolution of lignification have been based on the possibility that some algal ancestors of early land plants were washed ashore to provide populations on which Darwinian selection could operate (Bhattacharya and Medlin, 1998; Lewis and McCourt, 2004). The pioneering land plants would have faced many challenges including UV stress, desiccation stress, pathogen attack and lack of structural support, which may have led to the evolution of specialized metabolites through phenylpropanoid pathway (RAVEN, 1984; Bateman, et al., 1998). Lignin deposition in the cell walls is believed to coincide with the radiation of tracheophytes. Lignin not just reinforced tracheophtyes cell walls allowing for expansion of their body plan compared with their sister group, the bryophytes, but also facilitated long distance water transport (Kenrick and Crane, 1991; Uzal, et al., 2009; Graham, et al., 2000). With the co-evolution of pathogens and herbivores, lignin also augmented plant defense, making the cell walls resistant to enzymatic degradation, a selective advantage that aided in the early land plant adaptation (Boerjan, et al., 2003).

Lignin is a complex phenolic heteropolymer made up of *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, conferring mechanical strength, rigidity and hydrophobicity to the secondary cell walls of all vascular plants (Boerjan, et al., 2003). Just as other phenylpropanoid pathway products, lignin biosynthesis diverges from primary metabolism

with the deamination of amino acid phenylalanine to form cinnamic acid (Boerjan, et al., 2003; Fraser and Chapple, 2011). Following deamination, a series of hydroxylation and O-methylation reactions modify the propanoid side chain to form an alcohol moiety and to produce modified ring structures (Fraser and Chapple, 2011). This results in the production of the three most abundant lignin monomers or monolignols, which upon incorporation into the polymer are referred to as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units as shown in Figure 1 (Fraser and Chapple, 2011). Evidence also exists for an alternate route to formation of G and S lignin through 3-hyroxylation of caffeic acid and activation of corresponding co-A intermediate (Chen etal. 2011, Bassard etal. 2012). It was shown that poplar C4H and C3'H, two cytochrome p-450 monooxygenases, can interact to form a protein complex that can mediate hydroxylation reactions at the acid level and that either C4H or C3'H alone cannot catalyze this reaction (Chen etal. 2011). Similar C4H-C3'H protein associations was also reported in Arabidopsis (Bassard etal. 2012). After monomer synthesis in the cytoplasm, the monolignols are exported to the apoplast, where they undergo oxidation by peroxidases and laccases for polymerization into lignin in the secondary cell wall, thought to be mediated by radical coupling reactions between adjacent monolignols (Boerjan, et al., 2003; Wang, et al., 2013). The overall lignin polymer structure and physical properties may vary substantially between taxa and species owing to difference in composition and arrangement of the subunits (Bonawitz and Chapple, 2010). While H and G lignins are deposited in the cell wall of all the tracheophytes, S lignin is mostly found in Angiosperms and the lycophyte, Selaginella (Weng and Chapple, 2010). Gymnosperms primarily synthesize G-units with few H-units (Uzal, et

al., 2009). More H-units are found in monocots such as grasses, whereas, dicot lignins are mostly composed of G- and S-units (Boerjan, et al., 2003). The discovery of specialized cell walls and lignin in red alga, *Calliarthron cheilosporiodes*, revealed convergent evolution of the lignin biosynthetic scaffold in red alga and land plants (Martone, et al., 2009).

During monolignol biosynthesis, the aromatic ring undergoes deamination and hydroxylation at the first, second or third position followed by methylation of hydroxyl groups and subsequent reductions of propanoid side chain (Boerjan, et al., 2003; Fraser and Chapple, 2011), Figure 1. It is presumed that the enzymes required for lignin biosynthesis and deposition might have been acquired through either gene duplication events involving enzymes that are involved in the biosynthesis of primary metabolites or enzymes acquired via horizontal gene transfer from symbiotic organisms (Weng and Chapple, 2010; Tawfik, 2006; Richards, et al., 2006). It is possible that in a likely event of gene duplication, one of the newly derived genes can acquire certain selective advantages and therefore would have been selected for in the population (Lynch and Conery, 2000). Phenylalanine ammonia-lyase (PAL) functions at the beginning of the phenylpropanoid pathway by catalyzing the deamination of phenylalanine to ammonia and trans-cinnamic acid (Fraser and Chapple, 2011). PAL is both structurally and mechanistically similar to a primary metabolism enzyme involved in histidine degradation called histidine ammonia-lyase (HAL) (Ritter and Schulz, 2004; Baedeker and Schulz, 2002). PAL mirrors HAL in catalytic mechanism by adopting the co-factor 4-methylidene-imidazole-5-one group that helps to increase its electrophilicity (Ritter and Schulz, 2004; Baedeker and Schulz, 2002). Cinnamate 4-hydroxylase (C4H) and p-coumaroyl shikimate 3′-hydroxylase

(C3′H) are two cytochrome P450 monooxygenases that catalyze the initial hydroxylation reactions in the monolignol biosynthetic pathway (Weng and Chapple, 2010). While these enzymes are documented to share highly conserved heme binding motifs with sterol 14-demethylase, a primary metabolism enzyme, and also function through a common catalytic mechanism, they greatly deviate in their substrate recognition sites (Werck-Reichhart and Feyereisen, 2000). Extensive sequence homology is reported between 4-hydroxycinnamoyl-CoA ligase (4CL) and long-chain fatty acyl-CoA synthetase which is involved in the widely prevalent β-oxidation pathway (Weng and Chapple, 2010). A common catalytic mechanism that involves forming an acyl-adenylate intermediate is reported for these enzymes (Schneider, et al., 2003; Hisanaga, et al., 2004). 4CL is known to attach *p*-coumaric acid to coenzyme A (CoA) during biosynthesis of *p*-coumaroyl CoA, which is then channeled into either monolignol biosynthesis or flavonoid biosynthesis (Weng and Chapple, 2010). Hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT) is a member of the extensively diversified benzyl alcohol O-acetyltransferase anthocyanin O-hydroxycinnamoyltransferase N-hydroxycinnamoyl/benzoyltransferase deacetylvindoline 4-O-acetyltransferase (BAHD acyltransferase superfamily) (Weng and Chapple, 2010; D'Auria, 2006). HCT utilizes *p*-coumaroyl CoA as an acyl donor to synthesize *p*-coumaroyl shikimate, which is then utilized by C3′H during 3-hydroxylation reactions (D'Auria, 2006). Structural similarity is reported to exist between HCT and Carnitine acetyltransferase that transfers the acyl group of fatty acyl-CoA from coenzyme A to the isomer, L-carnitine, during the long chain fatty acid transport across mitochondrial membrane (Weng and Chapple, 2010). The

lignin biosynthetic pathway was recently revised to include caffeoyl shikimate esterase (CSE) which is involved in the hydrolysis of caffeoyl shikimate into caffeate and shikimate (Vanholme, et al., 2013). O-methyltransferases are required for G and S lignin biosynthesis (Whetten and Sederoff, 1995; Bonawitz and Chapple, 2010). Caffeoyl-CoA O-methyltransferase (CCoAOMT) utilizes S-adenosyl methionine (SAM) as the methyl donor to catalyze methylation of the hydroxyl group at the third position of caffeoyl CoA to yield feruloyl CoA (Whetten and Sederoff, 1995). Subsequent reduction of feruloyl CoA to coniferaldehyde by cinnamoyl-CoA reductase (CCR) and coniferaldehyde to coniferyl alcohol by cinnamyl-alcohol dehydrogenase (CAD) leads to G lignin (Humphreys and Chapple, 2002a). Caffeic acid 3-O-methyltransferase (COMT), on the other hand, mediates methylation of caffeylaldehyde for G lignin biosynthesis and 5-hydroxy coniferylaldehyde to produce S lignin (Guo, et al., 2001). The SAM binding sites are found in a highly conserved region, called α/β Rossmann fold, a structural motif found in nucleotide binding proteins. Mammalian catechol-O-methyl transferase transfers the methyl group from S-adenosyl methionine to catecholamines during degradation of neurotransmitters and is reported to share extensive structural and functional similarity with CCoAOMT (Ferrer, et al., 2005; Vidgren, et al., 1994). Ferulate 5-hydroxylase (F5H) is a P450-dependent monoxygenase that catalyzes the 5-hydroxylation of ferulic acid to 5-hydroxyferulic acid (Weng and Chapple, 2010; Meyer, et al., 1996). F5H is also reported to catalyze the formation of 5-hydroxyconiferylaldehyde from coniferaldehyde. F5H can also catalyze the formation of 5-hydroxyconiferyl alcohol directly by acting on coniferyl alcohol (Bonawitz and Chapple, 2010). Blocking the following

methylation step resulted in the deposition of a non-canonical lignin made up of 5-hydroxyguaiacyl unit derived from 5-hydroxyconiferyl alcohol (Hemm, et al., 2003). Hydroxycinnamoyl CoA thioesters are first reduced to hydroxycinnamaldehydes by (hydroxy) cinnamoyl-CoA reductase (CCR) and then to hydroxycinnamyl alcohols by (hydroxy)cinnamyl alcohol dehydrogenase (CAD) (Weng and Chapple, 2010). While CCR is similar to the mammalian steroid biosynthetic enzyme, 3-β-hydroxysteroid dehydrogenase, CAD shares extensive sequence homology with alcohol dehydrogenase commonly found in bacteria and animals (Lacombe, et al., 1997; Youn, et al., 2006).

The recalcitrant property of lignin not only confers mechanical strength to the plant cell walls but also provide protection from penetration by nutrient seeking pathogens (Hammond-Kosack and Jones, 1996). Some lignin biosynthetic genes have been reported to have distinct biochemical properties as a result of which, they may be specifically involved in plant defense rather than developmentally induced lignification (Miedes, et al., 2015). The possibility of defense induced, elicitor induced or pathogen triggered lignification in nature has been extensively reviewed (Miedes, et al., 2015). For example, there are two *CCR* genes in Arabidopsis genome. While, *CCR1* is expressed during developmentally regulated lignification, *CCR2* is only expressed upon bacterial infection (Lauvergeat, et al., 2001). Similarly, expression of an Arabidopsis *COMT* gene can be strongly induced upon treatment with a bacterial elicitor peptide, flg22 or necrosis-inducing *Phytophthora* protein 1 (NPP1) (Zipfel, et al., 2004). In some cases, elicitor treatment leads to the enrichment of certain lignin monomers over others. For instance, infection of wheat leaves with stem rust fungus led to the

increase of total lignin content but exclusively derived from syringyl alcohol (S-units) only (Menden, et al., 2007). In a similar study performed on tomato plants, H and G lignin was particularly enriched in the resistant lines through PAL expression, after inoculation with a fungal pathogen *Verticillium dahlia* (Gayoso, et al., 2010) and *Camelina sativa CCR2* is induced upon inoculation with *Sclerotina sclerotiorum*, resulting in G lignin accumulation (Eynck, et al., 2012).

1.2 Lignin engineering for efficient utilization of bioenergy feedstocks in biofuel production

As lignin is the major contributor to recalcitrance during cell-wall deconstruction for polysaccharide saccharification, attempts were undertaken to manipulate lignin quality and quantity by targeting the lignin biosynthetic enzymes (Chen and Dixon, 2007; Himmel, et al., 2007b; Vanholme, et al., 2010; Liu, et al., 2014; Humphreys and Chapple, 2002a; Li, et al., 2008b). The lignin biosynthetic enzymes and the steps catalyzed by them are shown in Figure 1. Modification of lignin biosynthesis pathway allows for bypassing the lengthy, costly and often harsh physical, chemical or thermal pretreatment steps required to remove lignin from bioenergy feedstocks (Kumar, et al., 2009; Mosier, et al., 2005). Almost every step of monolignol biosynthesis has been extensively studied in Arabidopsis and other species by either downregulation or overexpression of the enzyme involved (Vanholme, et al., 2010; Bonawitz and Chapple, 2010; Weng and Chapple, 2010). Alternatively, manipulation of some peroxidases and laccases, which are considered as targets for lignin polymerization, has been reported to improve cell wall saccharification (Berthet, et al., 2011; Herrero, et al., 2013;

Weng, et al., 2008; Lee, et al., 2013). Similarly, manipulation of transcription regulators belonging to NAC, MYB, bHLH and LIM family, involved in controlling certain aspect of lignification were also attempted to generate low lignin transgenic plants (McCarthy, et al., 2010; Zhong, et al., 2010a; Zhong, et al., 2010b). Progress has also been made by manipulating miRNAs involved in regulating lignification to study the consequence on lignin deposition, biomass yield and saccharification efficiency (Fu, et al., 2012; Rubinelli, et al., 2013). From the above biotechnological approaches, numerous gene targets have been identified that can be manipulated to reduce lignin and improve cell wall digestibility by cellulases to release sugars that can be fermented to bioethanol. However, perturbation of lignin biosynthesis often leads to undesirable pleiotropic effects (Bonawitz and Chapple, 2010) that will be discussed later in this chapter. Little is known about the molecular mechanisms mediating these morphological defects, necessitating research to identify the factors involved. Identification and characterization of these factors will not only improve our knowledge on how the process is sensed and mediated but also provide valuable targets for enhancing the quality of bioenergy feedstocks without compromising plant fitness.

1.2.1 Genetic modification of enzymes, transcription factors and miRNAs controlling lignification and consequent effect on plant growth

Lignification can be altered by targeting the monolignol biosynthetic genes, enzymes required for lignin polymerization, transcriptional regulators of lignification or regulatory factors affecting lignin biosynthesis (Yoon, et al., 2015; Poovaiah, et al., 2014). Several studies in Arabidopsis and other species have reported a negative correlation between lignin quantity

and release of fermentable sugars (Chen and Dixon, 2007; Van Acker, et al., 2014). While, transgenic lines obtained from downregulation of *HCT*, *C3'H*, *CCoAOMT*, and *CAD*, resulted in increased syringyl to guaiacyl (S/G) monolignol, the ones obtained from reducing *C4H*, *COMT*, *F5H* or *CCR* activity, resulted in decreased S/G ratio (Poovaiah, et al., 2014; Himmel, et al., 2007b). Except for *F5H*, downregulation of all other genes involved in lignin biosynthesis resulted in transgenic plants with increased cell wall digestibility (Li, et al., 2008a; Yoon, et al., 2015). *F5H* is specifically involved in S lignin biosynthesis and therefore is an important target for controlling the S/G ratio (Meyer, et al., 1996; Bonawitz and Chapple, 2010). *F5H* overexpressing Populus and Arabidopsis lines were reported to synthesize more S lignin, while the total lignin content was similar to wild-type plants (Ruegger, et al., 1999; Weng, et al., 2010; Stewart, et al., 2009). A strong correlation was observed between increased S lignin composition and enhanced saccharification efficiency upon hot water pretreatment (Li, et al., 2008a; Huntley, et al., 2003). While the change in S/G ratio is often considered as a parameter to assess the performance cell wall digestibility assay on pretreated biomass, this correlation is somewhat unclear due to conflicting reports on studies performed on different species. For example, while cell wall saccharification assay on some lignin downregulated alfalfa plants (Chen and Dixon, 2007), irrespective of with or without pretreatment did not support this claim, studies on Arabidopsis lignin mutants reported a negative influence of S/G ratio on sugar yield without prior pretreatment (Van Acker, et al., 2014).

PAL catalyzes the first step of phenylpropanoid biosynthesis and out of the four *PAL* genes in Arabidopsis, *PAL1* and *PAL2* play a major role in lignification (Raes, et al., 2003).

While high *PAL1*, *PAL2* and *PAL4* expression is observed in the vascular cells during late stem development and in the early stage of xylem differentiation during vascular development, low *PAL3* expression is reported in the stem (Mizutani, et al., 1997). Reduced lignin content with high S/G ratio is observed in *pal1 pal2* double mutant (Rohde, et al., 2004). Downregulation of all four Arabidopsis *PAL* genes reduces lignin levels to a large extent resulting in better saccharification efficiency (Huang, et al., 2010). However, several morphological anomalies like stunted growth, loss of pollen viability, changes in flower morphology, collapsed vasculature and dark green pigmentation in the leaves are also common in other species like tobacco and salvia (Bonawitz and Chapple, 2013; Song and Wang, 2011; Elkind, et al., 1990; Korth, et al., 2001). Downregulation of *C4H* in Arabidopsis has similar phenotypes like *PAL* quadruple mutants (Schilmiller, et al., 2009). Mutant alleles of *AtC4H* have distinct growth phenotype and S/G ratio, with the weakest allele exhibiting wild-type like growth with less lignin (Schilmiller, et al., 2009). *AtC3'H* downregulation results in hyper-accumulation of *p*-coumarate esters and flavonoids, reduced sinapoylmalate (a phenylpropanoid intermediate required for UV protection) and increased deposition of H-lignin units. The mutants exhibit severe dwarfism and vascular collapse (Franke, et al., 2002b). A range of mutant phenotypes with differences in effect on biomass yield as a result of *4CL* downregulation is reported in host species (Voelker etal., 2010; Anterola and Lewis 2002; Wagner etal., 2009; Li etal.,2015). For example, RNAi mediated *4CL* silencing in switchgrass results in browning of leaf mid-vein and stem internodes but can yield 57% more fermentable sugars compared to the control plants following an acid pretreatment (Xu and Cheng, 2011). Increased saccharification

efficiency was observed in Sorghum 4CL brown *midrib2* mutants, which show brown coloration in mid rib sclerenchyma tissues (Saballos, et al., 2008; Saballos, et al., 2012). While dwarfing is observed in *Pinus radiata 4CL* mutants (Wagner etal., 2009),*4CL* downregulated transgenic poplar lines reportedly have low lignin, increased cellulose content and enhanced growth (Voelker etal., 2010). Out of five rice *4CL* genes, *Os4CL3*, exhibits highest expression in lignifying vascular tissues and antisense *Os4CL3* lines are dwarf (Gui, et al., 2011). Out of the four 4CL isoforms found in Arabidopsis, *4CL1* is mainly involved in lignin biosynthesis (Li etal., 2015). With 70% of total lignin found in wild type, the *4cl1* mutant looks normal (Li etal., 2015). However, despite having similar lignin content as *4cl1* plants, the *4cl1 4cl2* and *4cl1 4cl2 4cl3* mutants are dwarf suggesting that reduction in lignin content and dwarfism are not always correlated (Li etal., 2015). Arabidopsis *CCoAoMT* mutants are reported to have no growth phenotype under long day conditions, but exhibit slightly reduced growth under short-day (Do, et al., 2007). *HCT* downregulated alfalfa plants are dwarf, have reduced total lignin, with very high H-units and decrease in both G and S units (Shadle, et al., 2007). Similar observations were made using recently identified Arabidopsis *cse* mutant (defective in Caffeoyl shikimate esterase), which exhibits slower growth but has lower total lignin level with higher proportion of H units and up to 75% increased saccharification efficiency over wild type (Vanholme, et al., 2013).

Changes in expression of genes acting in the monolignol pathway can directly impact the lignin content and/or lignin composition (Boerjan, et al., 2003). Downregulation of *CCR*, *COMT* and *CAD* reportedly increases saccharification efficiency. For instance, transgenic

poplar with downregulated *CCR* can release 50% more sugar relative to the control lines (Boudet, et al., 2003). In another independent study, *CCR* downregulation resulted in 161% more ethanol conversion but reduced growth was also observed. However, ethanol yield was still 57% higher than the non-transgenic line considering the yield penalty (Van Acker, et al., 2014). Arabidopsis *ccr1* mutants (known as *irx4*) are also dwarf with collapsed xylem, have dark green leaves with altered leaf morphology and 50% reduction in lignin (Jones, et al., 2001a). CCR-deficient Arabidopsis, tobacco and poplar plants are shown to incorporate free ferulic acid in their lignin (Ralph, et al., 2008). Antisense tobacco plants with reduced *CCR* activity have stunted growth, reduced lignin and collapsed xylem (Piquemal, et al., 1998). Transgenic poplar plants not only have reduced lignin but also altered pectin and hemicellulose deposition in their cell walls (Leple, et al., 2007). The Arabidopsis *F5H*-deficient *fah1* mutant, is defective in biosynthesis of S lignin and sinapoylmalate but exhibits wild-type growth (Ruegger, et al., 1999; García, et al., 2014). Similarly, downregulation of *COMT* is also characterized by decrease in S-lignin proportion as well as total lignin content. Interestingly, with suppressed *COMT* activity, a novel polymer derived from 5-hydroxyconiferylaldehyde is incorporated into lignin, which is thought to improve digestibility in transgenic lines (Simmons, et al., 2010). While COMT-deficient maize (*bm3*) and sorghum (*bmr12*) mutants exhibit moderate growth retardation (Oliver, et al., 2005; Lee and Brewbaker, 1984), no such effect was observed in the switchgrass and maize mutants (Fu, et al., 2011b; Piquemal, et al., 2002). In plants like Brachypodium, switchgrass and maize, reduced CAD activity resulted in

higher saccharification efficiency with moderate biomass loss (Saathoff, et al., 2011; Fornalé, et al., 2012; Bouvier d'Yvoire, et al., 2013; Fu, et al., 2011a).

Manipulation of laccases, candidates for catalyzing lignin polymerization are also considered potential targets for reducing lignin content and/or composition for improving saccharification efficiency (Vanholme, et al., 2008). For instance, Arabidopsis *lac4* and *lac17* mutants deposit less lignin. Interestingly, perturbation of both genes in *lac4 lac17* double mutants resulted in low lignin without any adverse growth defect (Berthet, et al., 2011). Although, these studies signify the importance of this attractive strategy in generating low lignin biomass, more research is required to evaluate the feasibility in field trials. Another emerging area of research is manipulation of transcription factors that regulate lignification. The lignin biosynthetic genes are generally regulated by NAC-MYB transcriptional cascades, where some act as activators and others as repressors (Yoon, et al., 2015; Castanet-Tolosan, 2012). NAC transcription factors act as master switches that activate MYBs (Castanet-Tolosan, 2012). For example, *SND1* and *SND2* are NAC family proteins shown to activate monolignol biosynthesis, while *VND1-7* act as master regulators of xylem cell differentiation (Poovaiah, et al., 2014; Castanet-Tolosan, 2012). Some R2R3 type MYB family proteins like Arabidopsis *MYB4*, switchgrass *MYB4*, Eucalyptus *MYB1* and maize *MYB31* act as repressors and Pinus MYB1, Eucalyptus MYB2 and poplar MYB3 act as activators of lignin biosynthesis (Poovaiah, et al., 2014). Another gene called Arabidopsis SHINE belonging to the AP2/ ERF TF family, was shown to regulate monolignol biosynthesis by regulating NAC and MYB proteins in a co-expression study (Ambavaram, et al., 2011). Overexpression of Arabidopsis

SHINE in rice led to dramatic decrease in G lignin with reduced total lignin content by 45%, and no obvious biomass loss (Ambavaram, et al., 2011). In another study, a repressor protein *PvMYB4* was overexpressed in switchgrass to see the effect on saccharification efficiency (Shen, et al., 2012). Although, the transgenic lines had low lignin and very high glucose yield efficiency, reduction in biomass was also observed. Involvement of regulatory RNA in the lignification process was recently reported (Fu, et al., 2012; Rubinelli, et al., 2013). For instance, in transgenic switchgrass, constitutive expression of the maize *Cg1* gene, encoding for *miR156* (a regulatory RNA), led to less lignin and 250% increase in starch content (Chuck, et al., 2011). However, overexpression of rice *miR156b* led to pleiotropic growth defects in low lignin switchgrass lines (Fu, et al., 2012).

1.2.2. Effects of alterations of lignin biosynthesis on phenylpropanoid metabolism

The suppression of lignin biosynthetic enzymes has been shown to alter the metabolic profile of the transgenic plants downregulated for the enzyme (Baxter and Stewart Jr, 2013). It is often hypothesized that upon perturbation, the flux may be re-directed from monolignol pathway to another pathway and consequently affect the biosynthesis and accumulation of other specialized metabolites. For instance, a case of "metabolic spillover" was hypothesized for Arabidopsis and alfalfa plants, downregulated for *HCT*, where flux is directed from the monolignol pathway to the flavonoid pathway through utilization of *p*-coumaroyl CoA by chalcone synthase (*CHS*) (Gallego- Giraldo, et al., 2011a; Besseau, et al., 2007). This redirection results in hyper-accumulation of anthocyanins revealed through HPLC profiling. Hyper-accumulation of soluble phenolic glucosides derived from *p*-coumaric acid, was

reported in transgenic poplar downregulated for *C3'H* (Coleman, et al., 2008a). Apparently, *p*-coumarates are potentially toxic and therefore, diverting flux to ester-linked glucosides may aid in mobilizing the toxic intermediates to the phloem or to the vacuole. Transgenic poplar and alfalfa plants downregulated for *CCoAOMT* were reported to accumulate altered levels of phenolic and caffeoyl glucosides due to redirected metabolic flux to sinapic acid (Chen, et al., 2003; Meyermans, et al., 2000). Feruloyl-CoA is the preferred substrate for *CCR* and is a potentially toxic compound. To avoid this toxic buildup upon reduced CCR activity, plants either hydrolyze feruloyl-CoA to ferulic acid and then form feruloylmalate or incorporate ferulic acid into the non-canonical lignin structure (Dauwe, et al., 2007; Leple, et al., 2007; Prashant, et al., 2011; Derikvand, et al., 2008). Arabidopsis *COMT* mutants hyper-accumulate hydroxyl feruloylmalate and is severely defective in sinapoylmalate production (Goujon, et al., 2003; Quentin, et al., 2009). *COMT* downregulation in switchgrass results in altered metabolite profile with hyper accumulation of ferulic acid, 5-hydroxy ferulic acid, vanillin, 5-hydroxy coniferaldehyde and a novel monolignol like metabolite called iso-sinapyl alcohol (Tschaplinski, et al., 2012). Downregulation of *CAD* in switchgrass, tobacco and flax leads to accumulation of coniferaldehyde, sinapyladehyde, ferulic acid, syringic acid and chlorogenic acid, suggesting that *CAD* downregulation may either result in accumulation of its substrate or divert the flux towards the shikimate pathway (Fu, et al., 2011a). R2R3 MYB transcription factors are reported to control many aspects of phenylpropanoid pathway including lignin biosynthesis. Overexpression of *ZmMYB42* in Arabidopsis, results in reduction of lignin as well as flavonoid and sinapoylmalate, suggesting that this protein negatively regulates both

lignin and flavonoid biosynthetic genes (Sonbol, et al., 2009). Reduced sinapoylmalate may be due to downregulation of genes directly involved in its biosynthesis like *C4H*, *F5H* or *COMT* or by targeting aldehyde dehydrogenase, which catalyzes the step towards sinapoylmalate production (Sonbol, et al., 2009). Overexpression of another maize transcription factor *ZmMYB31* in Arabidopsis, reduces lignin content and sinapoylmalate accumulation but induces flavonoid biosynthesis and over accumulates anthocyanins, suggesting channeling of flux from lignin to flavonoid biosynthesis (Fornalé, et al., 2010).

1.2.3. Effects of alterations of lignin biosynthesis on plant stress

Many of the plant specialized metabolites produced through phenylpropanoid pathway are involved in plant-environment interactions (Dixon and Paiva, 1995; Kliebenstein, 2004). It is conceivable that altered metabolite accumulation may influence plants' response to both biotic and abiotic stresses. Many stress response related and defense related genes were upregulated in transgenic lines with altered lignin biosynthesis in both Arabidopsis and other species (Gallego- Giraldo, et al., 2011b; Dauwe, et al., 2007; Leple, et al., 2007; Fornalé, et al., 2010). The mechanism that triggers this stress response is currently unknown. One hypothesis is that alteration of plant secondary cell walls may mimick pathogen damage or cell wall wounding, leading to release of polysaccharides, which are perceived as elicitors for defense responses, thereby triggering an adaptive response (Gallego- Giraldo, et al., 2011b; Dauwe, et al., 2007; Leple, et al., 2007). It is also proposed that misregulation of plant cell wall integrity maintenance system may constantly activate defense related genes, leading to altered accumulation of cell wall components and consequently affecting growth (Seifert and

Blaukopf, 2010; Hamann, 2012). Suppressed lignin biosynthesis can lead to altered phenolic levels in the mutants, which can affect plant-microbe interactions (Peters and Verma, 1990). For example, elevated levels of hydroxyl feruloyl malate, as a result of reduced *COMT* activity, enhanced resistance of Arabidopsis mutant lines to *Hyaloperonspora* (Quentin, et al., 2009). *COMT* reduced tobacco lines also developed fewer tumors when infected with *Agrobacterium* (Maury et al., 2010). Downregulation of *CCR* in *Arabidopsis thaliana* and poplar and *HCT* in poplar, was shown to upregulate genes encoding pathogenesis related (PR) proteins (Leple, et al., 2007). Elevated salicylic acid levels was also reported for transgenic alfalfa plants, which are known to trigger the expression of PR proteins (Gallego- Giraldo, et al., 2011b). Elevated levels of H_2O_2, an oxidative stress related phenotype, was reported in the leaves of poplar *CCR* mutants, that led to the hypothesis that plants may perceive the altered cell wall structure as a wound-like stress response and therefore leading to the upregulation of oxidative stress response genes (Leple, et al., 2007). Furthermore, detection of a phenolic compound called feruloyl tyramine at the site of wounding supported this hypothesis (Dauwe, et al., 2007). Upregulation of abiotic stress related transcripts, especially drought and heat stress related, were reported in *HCT* downregulated alfalfa plants (Gallego- Giraldo, et al., 2011b). Elevated ABA levels are also reported in transgenic plants, which are thought to activate drought sensitive genes (Gallego- Giraldo, et al., 2011b). Furthermore, differential accumulation of phenylpropanoid pathway intermediates such as the lignin precursors, *p*-coumaric acid, ferulic acid and sinapic acid may influence plant-microbe interactions (Barber, et al., 2000).

1.3 Little is known about the molecular mechanism mediating LMID

To summarize, severely lignin-depleted plants exhibit growth and developmental anomalies called lignin modification induced dwarfism (LMID). Notably, vascular collapse is widely observed in response to alteration of lignin biosynthetic genes earlier in the monolignol pathway. This defect is commonly attributed to the biological role of lignin as a structural component of cell wall that confers mechanical strength and help avoid cavitation during water transport (Li, et al., 2010; Kim, et al., 2014). In an attempt to rescue vascular collapse in *cse* and *c4h* low lignin mutants, the *CSE* and *C4H* genes were expressed under vessel specific promoters of transcription factors *VND6* and *VND7* respectively (Yamaguchi, et al., 2010a, Vargas etal. 2016). In contrast, a study reported the occurrence of dwarfism in *CAD* perturbed *F5H*-overexpressing lines with no evidence of collapsed xylem (Anderson, et al., 2015), suggesting the presence of alternative mechanisms. Another possibility for dwarfism is activation of defense pathways through salicylic acid mediated signaling as discussed earlier. A study conducted in alfalfa *HCT* down-regulated lines, suggested that hyper-accumulation of salicylic acid in lignin deficient plants may account for dwarfing (Gallego- Giraldo, et al., 2011b). Recently, it was shown that blocking salicylic acid synthesis did not rescue the severe dwarf stature of lignin deficient Arabidopsis *ref8* mutant which is known to hyper accumulate salicylic acid (Kim, et al., 2014). Hyper-accumulation of pathway intermediates like flavonoids was also reported leading to dwarfism in *HCT*-deficient plants through disruption in auxin transport (Besseau, et al., 2007). This claim was disproved using a dwarf *C3'H* mutant (*ref8*) and a *CHS* null mutant (*tt4-2),* where researchers were able to show that the growth phenotype of flavonoids lacking *ref8 tt4-2* double mutant is indistinguishable from that of *ref8* (Li, et al.,

2010). Defects arising from deficiency in *C3'H* in *ref8*, results in reduced total lignin with only H-lignin, blocked sinapate esters biosynthesis, hyper-accumulation of *p*-coumarates and stress induced-flavonoids, and dwarfism (Franke, et al., 2002a). Precise temporal control of *C3'H* gene expression using a chemically inducible gene expression system allowed researchers to investigate the link between dynamic changes of lignification, soluble phenylpropanoid accumulation and growth restoration suggesting that growth is dependent on the time of *C3'H* expression and subsequent lignification (Kim, et al., 2014; Li, et al., 2010). It is also conceivable that dwarfing in lignin biosynthetic mutants may be due to deficiency in the synthesis of a non-lignin metabolite like dehydrodiconiferyl alcohol glycosides for example, which have been shown to promote cell division in tobacco cell culture lines (Teutonico, et al., 1991). Therefore, a reduction in their activity might also contribute to the growth defects observed in phenylpropanoid pathway perturbed plants. However, this possibility is barely explored. Despite all these reasonable biochemical mechanisms that may be leading to dwarfing of lignin mutants, our knowledge of the molecular mechanisms mediating LMID remains incomplete and therefore, merits further investigation (Kim, et al., 2014; Bonawitz, et al., 2014a).

A recent report on the role of plant mediator subunits, *MED5a* and *MED5b* in transcriptional regulation of lignin biosynthetic genes was proposed (Bonawitz, et al., 2014a). It was demonstrated that *MED 5a/5b* are negative regulators of phenylpropanoid biosynthesis especially the lignin branch and mutation of *5a/5b* restored growth and other developmental anomalies in *ref8* through some currently unknown mechanism (Bonawitz, et al., 2014a).

Disruption of plant *MED5a/5b* subunits of the eukaryotic Mediator complex was able to restore LMID associated phenotypes of *ref8* by restoring total lignin to almost wild-type levels (Bonawitz, et al., 2014b). However, the lignin was almost entirely derived from H-units, a minor monolignol subunit *p*-coumaryl alcohol without restoring G and S. The soluble metabolite phenotype was also restored in *ref8 med5a/5b* plants. Together, these results demonstrate that LMID is a genetically controlled process.

1.4 Suppressor screen for investigation of LMID

LMID is a genetically controlled process and therefore, identification of suppressor genes that mitigate the growth defects of lignin-depleted mutants may be a powerful approach to understand the underlying genetic and cellular mechanisms. A suppressor screen through Ethyl methanesulfonate (EMS) mutagenesis is commonly used to identify second site modifiers, which reverse the phenotype associated with the primary mutation (Li and Zhang, 2016). This allows for identification of potential genes in the same or independent pathway contributing to the phenotype and thereby aids elucidation of molecular mechanism of the process of interest. For instance, a genetic screen that was carried out on *more axillary growth 2* (*max2*) mutant, exhibiting an enhanced seed dormancy phenotype led to the identification of the *max2* suppressor called *smaxl,* and subsequent characterization led to the discovery that SMAX1 is an important KARRIKINS/STRIGOLACTONE signaling pathway component involved in regulation of Arabidopsis seed germination and seedling growth (Stanga etal. 2013). Similarily, an important role of plant nucleoporins in hormone signaling was demonstrated by identification and characterization of the suppressors of Arabidopsis *auxin-resistant1* (*axr1*)

mutant called *sar1* and *sar3* (Parry etal. 2006). After identification of the suppressors, the mutants are backcrossed to the parent to generate mapping populations for identification of candidate mutations (Li and Zhang, 2016). Next generation sequencing based bulked segregant analysis is now used for identification of candidate genes, to bypass time consuming traditional map based cloning (Li and Zhang, 2016).

Identification of genetic pathways that contribute to the phenotypes of lignin mutants will be helpful in understanding what processes are affected upon lignin perturbation and how plants respond to this modification. Knocking out the same suppressor gene in another lignin mutant background will shed light if these genes have generic or specific roles in mediating LMID. Recently, it was postulated that "widespread transcriptional reprogramming" may attribute to the dwarfism in *ref8* mutant, which is defective in *C3'H* (Bonawitz, et al., 2014a). Investigation of the genetic components mediating LMID in the *C3'H* mutant will help in identifying the signal transduction components, which are recruited to mediate growth changes as an adaptive response to lignin modification. Identification of important factors including transcription factors and their downstream targets will not only elucidate the mechanism at the molecular level but also serve as new targets for biofuel feed stock improvement. It is also important to determine if LMID is caused by altered metabolite accumulation as a result of perturbed lignin biosynthesis. As altered phenolic profile may influence the way plants respond to plant stress, examination of connection between altered metabolite profile and plant growth is important to understand the role of phenylpropanoid metabolism in plant development. As hormones are reported to control every aspect of plant growth and development, investigation

of lignin mutants with altered hormone levels can also serve to connect the missing links. Finally, identification of Arabidopsis orthologs in bioenergy crops and translation of the knowledge obtained in Arabidopsis, will be necessary to access the effectiveness of the process and thereby generate low lignin biomass with enhanced saccharification and uncompromised growth that can be deployed in the field.

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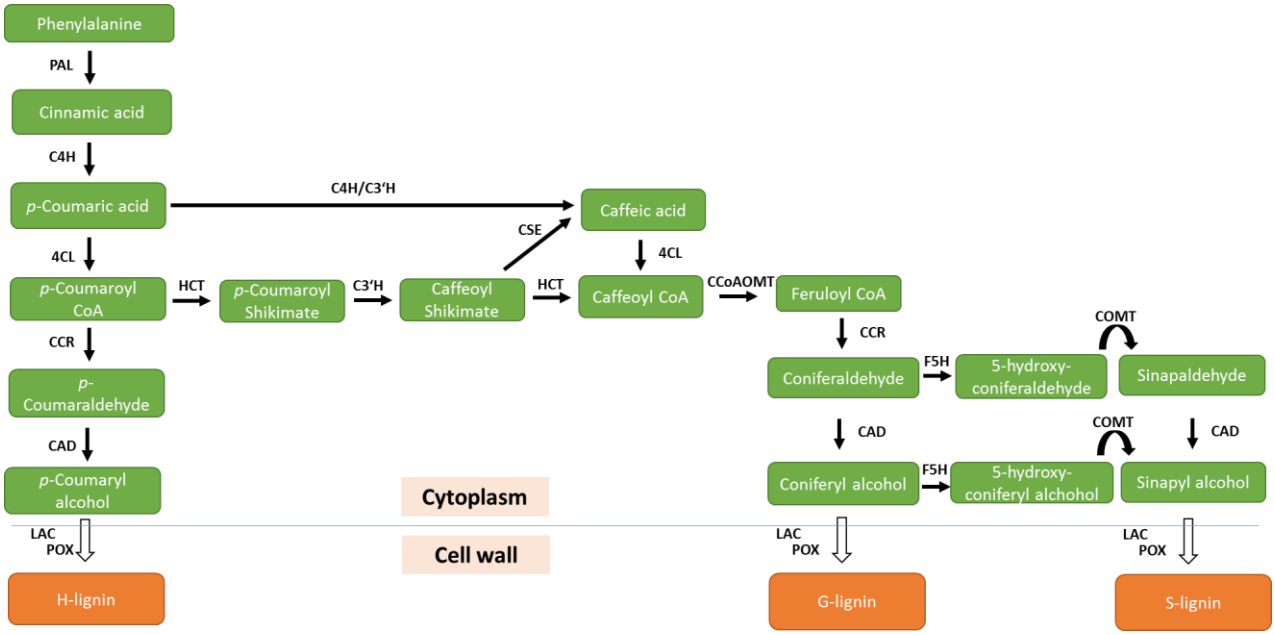

Figure 1: Simplified view of lignin biosynthesis pathway in Angiosperms starting from Phenylalanine. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; C3'H, p-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA O-methyl transferase; HCT, p-hydroxycinnamoyl-CoA shikimate hydroxycinnamoyl transferase;

CHAPTER 2

An importin-beta like protein mediates lignin modification induced dwarfism in Arabidopsis

ABSTRACT

Lignin modification induced dwarfism (LMID) imposes practical limitations to genetic enhancement of lignocellulosic biomass for biofuel production. Currently, little is known about the cellular and genetic mechanisms of LMID. Here we show that defects in both cell division and cell expansion underlie the dwarfism of the Arabidopsis lignin mutant *ref8*, and report the identification of a *GROWTH INHIBITION RELIEVED 1* (*GIR1*) gene from a suppressor screen. *GIR1* encodes an importin-beta like protein required for the nuclear import of MYB4, a transcriptional repressor of phenylpropanoid metabolism. Disruption of either *GIR1* or *MYB4* significantly alleviates the cellular defects and growth inhibition in *ref8*, but, surprisingly, does not upregulate the phenylpropanoid pathway, suggesting novel functions of *MYB4* distinct from its repressor role in phenylpropanoid metabolism. This discovery reveals key components of the transcriptional regulation circuits underlying LMID, opening doors to further elucidating the molecular pathways for this important phenomenon.

INTRODUCTION

Lignin is a highly complex phenolic polymer that confers mechanical strength, rigidity, and resistance of microbial degradation to secondary cell walls of vascular plants (Bonawitz and Chapple, 2010). The lignin polymer is formed by oxidative coupling of hydroxycinnamyl alcohol monomers, or monolignols, derived from the phenylpropanoid pathway (Ralph, et al., 2004). In angiosperms, polymerization of three major types of monolignols: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol gives rise to *p*-hydroxyphenol (H), guaiacyl (G), and syringyl (S) lignin units (Boerjan, et al., 2003; Bonawitz and Chapple, 2010). Multiple types of linkages between lignin monomers exist including aryl-ether (beta-*O*-4), phenylcoumaran (beta-5), and resionol (beta-beta) type linkages (Boerjan, et al., 2003; Bonawitz and Chapple, 2010). The heterogeneity of lignin polymer makes it a strong barrier to accessing cell wall polysaccharides and thereby limits utilization of lignocellulosic biomass for biofuel production (Li, et al., 2008). While genetic manipulation of lignin deposition is an attractive strategy to overcome this problem, significant reduction of biomass often came with severe growth defects and yield penalties (Bonawitz and Chapple, 2013; Li and Chapple, 2010; Chen and Dixon, 2007). Such lignin modification induced dwarfism (LMID) restricts the potential of cell wall engineering for biofuel crop improvement.

Genetic modification of various genes in the lignin biosynthetic pathway such as cinnamate 4-hydroxylase (*C4H*), *p*-coumaroyl shikimate 3′-hydroxylase (*C3′H*), hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (*HCT*), cinnamoyl-CoA reductase (*CCR*), and caffeoyl shikimate esterase (*CSE*) has been reported for a variety of plant

species (Franke, et al., 2002a; Jones, et al., 2001; Schilmiller, et al., 2009; Hoffmann, et al., 2004; Vanholme, et al., 2013; Ha, et al., 2016; Piquemal, et al., 1998; Leple, et al., 2007; Reddy, et al., 2005; Shadle, et al., 2007). Many of these lignin-modified plants are dwarf and have a collapsed xylem phenotype, suggesting that compromised water transport due to defective lignification of vessels may contribute to LMID. This is supported by the observations that Arabidopsis *ifl1* and *nst1 nst3* mutants, which have intact vascular vessels but no lignified interfascicular fibers, are not dwarf despite having limp inflorescence stems (Mitsuda, et al., 2007; Zhong, et al., 1997). However, restoration of vascular integrity in Arabidopsis *c4h* and *cse* mutants by driving the expression of *C4H* and *CSE* under the control of vessel-specific promoters only partially rescued the dwarfism (Vargas, et al., 2016; Yamaguchi, et al., 2010; Yang, et al., 2013), suggesting the presence of additional causes for growth inhibition.

Studies of biochemical changes in some lignin-modified plants have suggested other factors contribute to LMID. Hyperaccumulation of flavonoids was found in Arabidopsis HCT RNAi plants and was thought to be responsible for the reduced growth of the HCT-deficient plants through inhibition of auxin transport (Besseau, et al., 2007). However, a later study disproved this claim by demonstrating unequivocally that the dwarf phenotype of HCT-downregulated Arabidopsis plants is independent of flavonoid accumulation (Li, et al., 2010). In an alfalfa study, it was shown that accumulation of salicylic acid and jasmonic acid as well as mRNA levels of multiple pathogenesis-related (PR) genes were upregulated in HCT antisense lines, implying that HCT down-regulation leads to constitutive activation of defense

responses which is known to decrease plant growth (Gallego-Giraldo, et al., 2011; Huot, et al., 2014). However, blocking SA synthesis did not rescue the severe dwarf stature of Arabidopsis *c3′h* mutant (*ref8*) which also hyperaccumulates salicylic acid (Kim, et al., 2014). The stunted growth of *ref8* mutants could not be rescued by blocking flavonoid biosynthesis, either (Li, et al., 2010). A recent discovery revealed the involvement of two specific subunits of the transcriptional co-regulatory Mediator complex in the LMID exhibited by *ref8*, but the molecular mechanism remains unknown (Bonawitz, et al., 2014).

Plant growth and development involves integration of genetic and environmental information at the cellular level. Several interconnected processes such as cell division, expansion, and differentiation orchestrate at different rates and durations to control final leaf size (Gonzalez, et al., 2012). A possible connection between lignin pathway perturbation and changes in cellular processes was suggested by a recent study on the Arabidopsis *ccr1* mutant. It has been reported that hyperaccumulation of ferulic acid due to disruption of *CCR1* leads to delayed exit from cell proliferation during leaf development (Xue, et al., 2015). However, the cellular defects underlying growth in other lignin-modified plants are largely unknown.

The *ref8* mutants are among the most severe LMID mutants in Arabidopsis. The original *ref8* mutant (*ref8-1*) has a missense mutation in *C3′H* that significant reduces but does not abolish its enzyme activity, yet the plants are smaller than the *cse* or *ccr* null mutants and sterile (Franke, et al., 2002b; Franke, et al., 2002a). T-DNA insertional *ref8* null mutants stop growing after emergence of a few extremely small leaves (Weng, et al., 2010; Abdulrazzak, et al., 2006). Previously, we generated a *ref8*^{pOpON} line that can be conditionally complemented

by chemically inducible expression of a wild-type *C3'H* transgene (Kim, et al., 2014). This allowed us to perform a suppressor screen to identify the genetic components of LMID. Here, we report the isolation of suppressor lines that grow significantly bigger than the parental *ref8* mutant and the identification of the *GROWTH INHIBITION RELIEVED 1* (*GIR1*) gene. *GIR1* has been previously reported to encode an importin-beta protein that mediates the transport of MYB4 transcription factor from the cytosol into the nucleus (Zhao, et al., 2007). We discovered by kinematic analysis that *ref8* is severely defective in both cell division and cell expansion and these defects are alleviated in the suppressor plants. Our data also show that disruption of *MYB4* has similar rescue effects as *gir1* on *ref8* dwarfing, indicating that *GIR1* acts through *MYB4*. In contrast to the previous Mediator study (Bonawitz, et al., 2014), the growth restoration by *gir1* or *myb4* was not associated with upregulation of the phenylpropanoid pathway. Therefore, our work reveals distinct components in transcriptional regulation of LMID and suggests additional functions of MYB4 beyond phenylpropanoid pathway repression.

RESULTS

Isolation of suppressor mutants for *ref8* dwarfism

To identify genetic factors underlying LMID in the Arabidopsis *ref8* mutant, we carried out a suppressor screen to look for mutants that show an alleviated growth phenotype. Because *ref8* is sterile, the *ref8*^{pOpON} line carrying a chemically inducible *C3'H* expression construct (Kim, et al., 2014) was used to generate seeds for mutagenesis. When sprayed with dexamethasone, *ref8*^{pOpON} grows significantly better and becomes fertile, whereas in the absence of dexamethasone, *ref8*^{pOpON} behaves the same as the original *ref8* mutant (Kim, et al., 2014). Seeds of *ref8*^{pOpON} were mutagenized with ethyl methanesulfonate (EMS) and M1 plants were induced with dexamethasone to obtain M2 seeds. Screening 180 M2 pools led to the identification of more than 30 independent suppressor lines that showed significant growth restoration in the absence of dexamethasone (Fig. 1).

Identification of the *GROWTH INHIBITION RELIEVED 1* (*GIR1*) gene

We combined next-generation sequencing with bulked segregant analysis (Michelmore, et al., 1991) to map the suppressor genes, designated as *GROWTH INHIBITION RELIEVED* (*GIR*). A recessive suppressor mutant was backcrossed to the original *ref8*^{pOpON} mutant for generating F2 mapping population. Genomic DNAs were extracted from two F2 bulks generated by pooling individual plants based on their growth phenotype for Illumina sequencing. Mutations and their allele frequency in each of the two bulks were obtained by the Arabidopsis Col-0 genome sequence comparison. The mutations that were homozygous in the suppressor-looking bulk and had an allele frequency of approximately one-third in the *ref8*-

looking bulk were identified as putative causal mutations. By this approach, we mapped the mutations from five independent suppressor lines to a single gene, *GIR1* (Fig. 2A). Three of the five *gir1* alleles had a nonsense mutation that introduces a premature stop codon, and the other two alleles had a mutation at the 3′ end of an intron, which is likely to affect splicing.

To confirm that *GIR1* inactivation suppresses *ref8* dwarfism, we carried out a functional complementation test by transferring wild-type *GIR1* into the genome of the suppressor line. A reversal to dwarf phenotype was observed (Fig. 2B). In addition, we obtained a T-DNA knockout line of *GIR1* (*gir1-t*) from the Arabidopsis Biological Resource Center (Alonso, et al., 2003) and crossed it with $ref8^{pOpON}$ (Fig. 2A). The double mutant, *ref8 gir1-t* exhibited an enlarged rosette size, similar to the suppressor plants (Fig. 2C). These results demonstrate that *GIR1* is indeed the causal gene responsible for mediating dwarfism in *ref8*. Disruption of *GIR1* also alleviated the growth phenotype of the T-DNA knockout mutant, *ref8-t* (Fig. 2D), suggesting that the growth rescue is independent of C3′H activity.

Cellular changes underlying *ref8* dwarfism and the effects of *gir1* mutation

As an initial step to charactering the growth alleviation effect of *gir1*, we examined closely the growth of wild-type, *ref8*, and *ref8 gir1* seedlings. As early as two days after planting (DAP), *ref8* could be distinguished from wild type by its smaller cotyledons, and the size differences increased as plants grew, with *ref8* cotyledons being only half of the wild-type size by 10 DAP (Figs. 3A and 3B). The cotyledons of *ref8 gir1* were initially similar to wild type in size but their growth was apparently slower than wild-type cotyledons at later stages (Figs. 3A and 3B). These observations indicate that the growth inhibition in *ref8* as well as its

alleviation by *gir1* starts at a very early developmental stage, probably during germination. Leaf growth was monitored by leaf area measurement based on the first pair of leaves (Figs. 3A and 3C). Similar to the cotyledon results, *ref8* leaves remained smaller than wild-type leaves from emergence to maturation with the size differences increasing as plants grew. The suppressor leaves also showed early growth inhibition, but they underwent increased growth after 10 DAP, in contrast to *ref8*, which remained stagnant (Fig. 3C).

In order to understand the cellular basis of growth inhibition in *ref8* and its alleviation in *ref8 gir1*, we tracked epidermal cell growth by measuring the number and size of pavement and guard cells at multiple time points throughout leaf development. The first true leaf pair (leaves 1 and 2) was harvested and analyzed. In wild-type leaves, both the number and size of the cells increased from 6 DAP to 10 DAP, after which the cell number ceased increasing but cell size continued to increase (Figs. 3D and 3E). Due to the technical difficulty imposed by the small size of leaves from young seedlings of the *ref8* and suppressor plants, the kinematic analysis for these plants started at 8 DAP. In *ref8*, there were fewer cells than in wild type at 8 DAP and the cell number remained unchanged throughout the time points measured, suggesting that cell division was defective (Fig. 3D). In addition, *ref8* had smaller cells than wild type at 8 DAP, and cell size increased minimally during development, indicating reduced cell expansion in this mutant (Fig. 3E). While the suppressor plants (*ref8 gir1*) had a similar cell count as *ref8* early in development, its cell number increased significantly at 10 DAP and nearly reached wild-type levels at 18 DAP (Fig. 3D). Similarly, cell size in the suppressor plants was close to *ref8* at the beginning, but became significantly bigger than *ref8* at 10 DAP

(Figure 3E). We also calculated the stomatal index, which is described as the percentage of guard cells in all epidermal cells per unit leaf area (Asl, et al., 2011). In contrast to the clear differences in cell number and cell size, the stomatal indices of all genotypes were similar overall, albeit some fluctuations at different time points (Fig. 3F). This suggests that the *ref8* and *gir1* mutations have little effect on guard cell differentiation.

The effects of *gir1* on lignin and soluble phenylpropanoid phenotypes

In addition to dwarfism, deficiency of C3′H in *ref8* results in multiple changes in phenylpropanoid metabolism including reduced lignification, altered lignin composition, blocked sinapate ester biosynthesis, and hyperaccumulation of *p*-coumarate esters and flavonoids (Franke, et al., 2002a; Li, et al., 2010). To further characterize the *ref8* suppressor, we compared the biochemical phenotypes of the wild-type, *ref8*, and *ref8 gir1* plants. We analyzed cell wall lignification by phloroglucinol staining of cross sections of inflorescence stems (Fig. 4A). Consistent with previous reports (Kim, et al., 2014; Weng, et al., 2010), in wild-type plants the vascular bundles and interfascicular fibers were heavily lignified, as indicated by dark red staining (Fig. ?), whereas the weak staining of *ref8* sections reflected a reduction in lignification. The *ref8 gir1* sections showed more intense staining compared with *ref8*, suggesting increased lignification in the suppressor plants. Notably, although not as severe as in *ref8*, the vascular tissues were still deformed in the *ref8 gir1* plants (Fig. 4A). Determination of total lignin content by the acetyl bromide assay (Chang, et al., 2008) showed that lignin levels in inflorescence stems was partially restored in *ref8 gir1* compared with *ref8* (Fig. 4B), consistent with the histological staining results. Lignin composition analysis using

the Derivatization Followed by Reductive Cleavage (DFRC) method (Lu and Ralph, 1998) revealed that the deficiency of G and S lignin observed in *ref8* was not restored in the suppressor plants and the increased lignification seemed to be mainly due to enhanced H lignin biosynthesis (Fig. 4C). The relative abundance of lignin in *ref8* and *ref8 gir1* compared with wild type was lower in the DFRC than in the acetyl bromide measurement because DFRC specifically quantifies lignin monomers released from beta-*O*-4 linkages (Lu and Ralph, 1998) and *ref8* has been shown to contain a substantially lower proportion of beta-*O*-4 linkages than wild type (Bonawitz, et al., 2014).

We also measured soluble phenylpropanoid phenotypes. It is well known that *ref8* has a phenylpropanoid profile distinct from wild type, including presence of *p*-coumarate esters, increased flavonoid accumulation, and decreased levels of sinapate esters (Franke, et al., 2002a; Li, et al., 2010). Our results showed that *ref8 gir1* has a similar profile as *ref8* (Fig. 4D), suggesting C3′H deficiency is not alleviated in the suppressor plants.

Testing the involvement of MYB4 in *ref8* dwarfism

Since *GIR1* has been previously reported to encode an importin-beta protein that mediates the nuclear import of MYB4, a negative regulator of the phenylpropanoid pathway (Zhao, et al., 2007; Jin, et al., 2000), we tested if GIR1 functions in LMID via MYB4 by investigating the effect of MYB4 loss-of-function on *ref8* dwarfism. Because *ref8* was generated from Col-0 and there were no available *myb4* mutants with the same genetic background, we used the newly developed CRISPR/CAS9 genome editing technique (Sander and Joung, 2014) to specifically mutate the *MYB4* gene in Col-0. We isolated a mutant line

that has a 2-bp deletion within the first exon, which introduces a frameshift mutation leading to a premature stop codon in the *MYB4* gene. This mutant accumulated higher levels of sinapate esters than wild type (Fig. 5B), consistent with the previous reports of *myb4* mutants (Zhao, et al., 2007; Jin, et al., 2000).

To test if MYB4 mediates *ref8* dwarfing, we crossed the *myb4* mutant with *ref8*^{pOpON}. Out of a population of 114 F2 plants, 85 plants showed wild-type growth, 19 plants were *ref8*-like, and 10 plants looked like suppressors. These data are consistent with the expected 12:3:1 segregation ratio for wild type: *ref8: ref8 myb4* (χ^2 test, *p*-value = 0.5). Sequencing and genotyping of five suppressor-looking plants confirmed that they were all *ref8 myb4* double mutants. These results demonstrate that MYB4 mediates the dwarfism of *ref8* (Fig. 5A). Importantly, side-to-side comparison revealed that *ref8 myb4* showed a similar extent of growth alleviation as *ref8 gir1* (Fig. 5A). We also compared cellular changes of these plants. Kinematic analysis of leaves from 18 day old plants revealed that *myb4* rescued the cellular defects of *ref8* to similar levels as *gir1* (Figs. 6A and 6B). These results suggest that the effect of GIR1 on *ref8* dwarfism is largely exerted through MYB4.

To test if GIR1 and MYB4 are involved in controlling leaf growth and development in wild type, we performed a kinematic analysis on the *gir1* and *myb4* single mutants. In contrast to the large effects observed in the *ref8* background, there were no statistically significant differences in either cell number or cell size between wild type, *gir1*, and *myb4* (Figs. S1A and S1B). These plants also had a similar stomatal index (Fig. S1C).

It is well known that MYB4 represses the expression of the key lignin biosynthetic gene, *C4H* (Zhao, et al., 2007; Jin, et al., 2000). In order to gain insight into how MYB4 is involved in LMID, we measured *C4H* expression in young seedlings by qRT-PCR. *C4H* expression levels in *ref8 myb4* and *ref8 gir1* were found to be similar to that of *ref8* plants (Fig. 6C). Consistent with this observation, these plants also showed similar levels of accumulation of sinapate esters, *p*-coumarate esters, and flavonoids (Fig. 5B). Taken together, these results indicate that phenylpropanoid metabolism is not upregulated in *ref8 myb4* compared with *ref8* and that both *gir1* and *myb4* have similar effects on cell number and cell size in *ref8 gir1* and *ref8 myb4* respectively.

DISCUSSION

LMID is a major hurdle for genetic improvement of biofuel crops through lignin engineering. Currently, there is very little knowledge available regarding the cellular and genetic mechanisms of this important phenomenon. In this study, by using the Arabidopsis *ref8* mutant, we carried out a suppressor screen to identify genetic components of LMID. We successfully isolated dozens of suppressor lines that grow significantly better than the parental *ref8* mutant. These lines are valuable resources for identifying genes and pathways involved in mediating the growth effect of phenylpropanoid pathway perturbation.

We have demonstrated that mutations in *GIR1* partially rescue *ref8* dwarfism. The *GIR1* gene had been previously identified in several different genetic screens. As revealed from these studies, loss of function of *GIR1* results in pleotropic phenotypes including hypersensitivity to ABA (Verslues, et al., 2006), enhanced UV resistance (Zhao, et al., 2007), reduced trichome number (Gao, et al., 2008; Yoshida, et al., 2009), and enhanced miRNA activities (Wang, et al., 2011). As *GIR1* encodes an importin-beta family protein that mediates the transport of proteins or RNAs from the cytosol to the nucleus through nuclear pore complexes (Cautain, et al., 2015), the phenotypes of *gir1* likely reflect the functions of its cargo molecules. It has been reported that GIR1 is required for nuclear transport of several R2R3 MYB transcription factors including MYB4 (Zhao, et al., 2007; Zhou, et al., 2015), a transcriptional repressor of the phenylpropanoid pathway (Jin, et al., 2000). This connection prompted us to test the possibility that MYB4 is involved in LMID. Indeed, the *ref8 myb4* double mutant grows significantly bigger than *ref8*, supporting the idea that GIR1 mediates

LMID through MYB4. The phenotypic similarity between *ref8 gir1* and *ref8 myb4* suggests that other cargos of GIR1 have little or no relevance to *ref8* dwarfism.

As plant growth involves multiple spatially and temporally coordinated cellular processes, it is important to investigate the specific cellular changes underlying LMID. After leaf initiation, leaf growth mainly depends on two sequential processes, cell proliferation and cell expansion, that occur successively throughout the cell population in a basipetal direction (Beemster, et al., 2003; Beemster, et al., 2005; Beemster, et al., 2006; Gonzalez, et al., 2012). We found that *ref8* has fewer and smaller leaf cells suggesting that both cell proliferation and expansion are affected in this mutant. This is in contrast to the *ccr1* mutant, which was reported to have more but smaller cells due to delayed exit from the cell proliferation phase (Xue, et al., 2015). Therefore, LMID in *ref8* and *ccr1* is likely due to different mechanisms, although both mutants have the collapsed xylem phenotype (Jones, et al., 2001; Franke, et al., 2002a).

Since plant hormones are important growth regulators and many hormone mutants show cell proliferation and expansion defects, our kinematic analysis results provide clues to possible connections between LMID and hormone metabolism/signaling. The cellular phenotype of *ref8, i.e.*, reduction of both cell number and cell size, has also been observed in several ABA, auxin, or ethylene mutants (Horiguchi, et al., 2006), suggesting that these hormones may be misregulated in *ref8*. Notably, the *auxin response factor 2* and *ethylene response 1* mutants were reported to have more and bigger cells than wild type (Horiguchi, et al., 2006). It would be interesting to investigate if *gir1* or *myb4* alleviates *ref8* growth by regulating these genes.

Our discovery that disruption of GIR1 or its cargo MYB4 alleviates the dwarfism and cellular defects of *ref8* suggests active transcriptional regulation in LMID. This is consistent with the previous report that thousands of genes are differentially expressed in *ref8* compared with wild type (Bonawitz, et al., 2014). These large-scale transcriptional changes were shown to be dependent on the MED5a and MED5b subunits of Mediator complex, a transcriptional coregulator (Bonawitz, et al., 2014). Interestingly, although both MED5a/5b and GIR1/MYB4 are known to repress phenylpropanoid metabolism in wild type, upregulation of the phenylpropanoid pathway was only observed in the *med5a*/*5b ref8* triple mutant but not in *ref8 gir1* or *ref8 myb4*. This suggests that GIR1 and MYB4 may have LMID-specific roles in a pathway distinct from that involving MED5a/5b.

Considering that perturbation of lignin pathway may trigger activation of stress responsive genes (Gallego- Giraldo, et al., 2011; Dauwe, et al., 2007; Leple, et al., 2007; Hamann, 2012) and the well-known involvement of R2R3 type MYBs in various defense and stress related responses (Dubos, et al., 2010; Saibo, et al., 2009), it is conceivable that MYB4 is recruited in response to the signal generated from lignin modification, such as water deficit, cell wall integrity breach, or some phenylpropanoid intermediates, to activate a chain of events that lead to repression of cell proliferation and expansion, a plant adaptive response ultimately manifested as stunted growth. Some R2R3 type MYB transcription factors have been implicated in regulation of cell proliferation and cell cycle progression. For example, Arabidopsis *myb11* mutant has reduced meristematic cell proliferation activity and MYB11 overexpressing lines show the opposite phenotype (Petroni, et al., 2008). Arabidopsis MYB59

and MYB125 have been reported to regulate cell division in root and germline cells respectively (Brownfield, et al., 2009; Mu, et al., 2009). Therefore, it is possible that MYB4 may also regulate gene expression in similar cellular processes. An immediate next step would be to identify downstream targets of MYB4 in a *ref8* background and test their relevance to the dwarf phenotype. It is expected that identifying other *GIR* genes from our collection of *ref8* suppressor lines and studying their relationship in the near future would greatly facilitate the elucidation of molecular mechanisms underlying LMID.

MATERIALS AND METHODS

Plant material and growth conditions

Scotts Osmocote Plus slow release fertilizer (Hummert International, Earth City, MO) was added to Redi-earth Plug and Seedling Mix (Sun Gro Horticulture, Agawam, MA) for growing *Arabidopsis thaliana* plants in a growth chamber. A 16-h light/8-h dark photoperiod was used with a 100 μE m^{-2} sec^{-1} light intensity at 21°C. The Arabidopsis *ref8-1* and *ref8^{popOn}* lines have been explained previously (Franke, et al., 2002b; Kim, et al., 2014). The T-DNA insertional mutants *gir1-t* (SALK_133577C) and *ref8-t* (SALK_112823) were obtained from the Arabidopsis Biological Resource Center. The wild type and all mutants are in the Col-0 background.

Suppressor screening

EMS mutagenesis of *ref8^{popOn}* was carried out following an established protocol (Weigel and Glazebrook, 2006). Approximately 125,000 seeds were mutagenized in a 0.2% (v/v) EMS solution (Sigma, St Louis, MO, USA) for 16 h. After extensive rinsing with water, the seeds were planted in soil. M1 plants were sprayed with 20 μM dexamethasone containing 0.02% Silwet L-77 once a week to allow bolting and setting seeds (Kim, et al., 2014). M2 seeds were harvested from 180 pools of M1 plants with approximately 350 plants in each pool. M2 plants were grown in the absence of dexamethasone and the plants displaying restored growth were isolated as putative suppressors. The growth phenotype of the suppressor lines and the presence of the original *ref8* mutation were confirmed in the M3 generation.

Mapping of *GIR1*

The suppressor and *ref8*^{popOn} lines were grown with dexamethasone treatment and crossed. The F1 plants were selfed to produce F2 seeds and the F2 plants were grown in the absence of dexamethasone. Approximately equal amount of leaf tissue from each of fifty to one hundred individual plants that showed the same growth phenotype, either *ref8* or suppressor, was harvested and pooled together, resulting in two bulks. Genomic DNA was extracted from the bulked tissue using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD). Pair-end genomic libraries were constructed following the Illumina TruSeq genomic DNA library preparation protocol. Illumina HiSeq2000 was used for sequencing. Approximately 15 G raw data (100 bp pair-end sequences) were generated for each sample.

The sequence reads were mapped to the TAIR10 Arabidopsis reference genome using BWA v0.7.10 (Li and Durbin, 2009). The output alignment was further refined by indel realignment using GATK v3.3 (McKenna, et al., 2010) and removal of PCR duplicates using Picard v1.127 (http://broadinstitute.github.io/picard). The final bam files from the *ref8* bulk and the suppressor bulk were used as input to GATK for joint calling of variants (DePristo, et al., 2011). An in-house R script was used to extract the variant information and calculate the allele frequency of a variant in each of the two bulks to identify the putative causal mutation.

Functional complementation

The *GIR1* gene was amplified from Col-0 with primers oXL1086 (5′-GCACTGATTTGAAAAATCTCAGGGAATTTCGTGGATTGGAGA-3′) and oXL1087 (5′-CCGGGTCTTAATTAACTCTTTTGTCTCTTGAATGCCTGCTACT-3′), and cloned into pEarleyGate103 using the Gibson Assembly Cloning Kit (New England BioLabs, Ipswich,

MA). This construct (pXL1003) was transferred into *ref8 gir1* via *Agrobacterium tumefaciens*–mediated transformation (Clough and Bent, 1998). Ten T2 lines were isolated by glufosinate ammonium selection and they all show *ref8* growth phenotype.

Genotyping

Primer sequences used for genotyping are shown in Table S1. The primers oXL1001 and oXL1002 were used in combination with *Eco*RV digestion for genotyping the endogenous *REF8* locus, as previously described (Kim, et al., 2014). The wild-type allele gives rise to a 865-bp PCR product that is resistant to *Eco*RV digestion, whereas the amplicon from the *ref8-1* allele is cleaved into two fragments (584 bp and 281 bp). Similarly, oXL1041 and oXL1042 were used with Hpy166II to detect the *gir1-1* allele (wild type: 488 bp; *gir1-1*: 357 bp and 131 bp). For T-DNA mutants, the wild-type allele is detected by PCR using a pair of gene-specific primers, LP and RP; and the mutant allele is detected by amplification with a T-DNA border primer (BP, oXL1003) (Alonso, et al., 2003). Primers oXL1034 (LP) and oXL1035 (RP) were used for genotyping *gir1-t*. Primers oXL1242 (LP) and oXL1241 (RP) were used for *ref8-2*.

Histochemical staining of stem sections

The base of Arabidopsis primary inflorescence stems were embedded in 5% agarose and cut into 50-μm sections using a PELCO easiSlicer vibratome (Ted Pella, Inc., Redding, CA). The stem cross sections were stained with 1% (w/v) phloroglucinol solution that contains 10% (v/v) HCl and 50% (v/v) ethanol for 10 min, then mounted in water and visualized with a Carl Zeiss light microscope. The images were taken using an AxioCam ICc 5 camera and analyzed using the software AxioVision SE64 Rel. 4.9.1.

Lignin Analysis

Eight weeks old Arabidopsis inflorescence stems were stripped of all cauline leaves and siliques, cut into small pieces, and finely ground in liquid nitrogen. Ground tissue was then subjected to an extraction in ten volumes of 0.1 M sodium phosphate buffer (pH 7.2) at 50 °C for 1 h, six extractions in ten volumes of 70% ethanol (v/v) at 70 °C for 15 min, and a final extraction in 100% acetone for 10 min. Cell wall residue was then collected by centrifugation and dried overnight.

The prepared cell wall materials were used for lignin analysis. DFRC was performed following a previously reported procedure (Li, et al., 2010). The lignin monomer derivatives were analyzed on an Agilent GC 7820A with a flame ionization detector. The relative response factors to the internal standard 4'-ethylidenebisphenol (by weight) were determined to be 0.65, 0.72, and 0.53 for H, G, and S monomers, respectively. Total lignin content was determined by the acetyl bromide assay. Two to five mg of cell wall material was dissolved overnight in 2.5 mL 25% (v/v) acetyl bromide in glacial acetic acid. After all cell wall material is dissolved, the cell wall solution is transferred to a 10 mL volumetric flask containing 2 mL 2 M NaOH and subsequently mixed with 0.35 mL of freshly made 0.5 M hydroxylamine HCl solution. After adding acetic acid to bring up the volume to exact 10 mL mark, the absorbance at 280 nm was measure for the solution using a spectrophotometer. Lignin content was calculated using the reported extinction coefficient (23.29 g^{-1} L cm^{-1}) for Arabidopsis Col-0 (Chang, et al., 2008).

HPLC analysis of soluble phenylpropanoids

Rosette leaves were harvested and extracted with 50% (v/v) methanol at 60°C for 30 min. Samples are centrifuged at 14,000 g for 5 min before loading on HPLC. Five microliters of extract was separated on an Eclipse plus C18 column (3- × 100-mm with 1.8 µM particles) from Agilent using 0.1% (v/v) formic acid in water as solvent A and 0.1% (v/v) formic acid in acetonitrile as solvent B. The soluble phenylpropanoids were separated by a gradient over 28 min, starting with a hold at 2% solvent B for 1 min, followed by an increase of gradient to 20% B over 20 min, and then to 90% B over 1 min and hold at 90% B for 2 min. Data were recorded at 330 nm with a spectral bandwidth of 4 nm. Peak identification and quantification was done as described previously (Kim, et al., 2014).

Generation of the myb4 mutant using CRISPR/Cas9 system

A 20-nucleotide sequence (5′-GTCGCCTACATTAAAGCTCA-3′) was selected as a gRNA target site specific to Arabidopsis *MYB4* from the CRISPR-PLANT database (Xie, et al., 2014). A sgRNA expression cassette that contains Arabidopsis U6-26 promoter driving this gRNA target sequence was synthesized as a gBlocks from Integrated DNA Technologies, Inc. (Coralville, IA). This cassette was inserted into the pFGC-pcoCas9 (Addgene plasmid # 52256, Cambridge, MA) linearized with *Asc*I using the Gibson Assembly Cloning Kit (New England BioLabs, Ipswich, MA). The resulting construct (pXL1023) was transformed into *Agrobacterium tumefaciens* by electroporation, and then introduced into Arabidopsis Col-0 plants by the floral dip method (Clough and Bent, 1998). Transgenic lines were selected by glufosinate ammonium. T2 and T3 plants were screened for mutations by sequencing the

MYB4 region amplified with oXL1182 (5′-ACCCTCGCTAAAAGCCAATC-3′) and oXL1183 (5′-TTCATGGAACGTTTCGACCT-3′).

Kinematic analysis

The whole leaf images were taken using a Zeiss Stereoscope Discovery V20 mounted with PlanApo S 1.0x FWD 60 mm lens. The total area of the leaf was calculated using the curve spline feature under the area measurement tab in the software AxioVision SE64 Rel. 4.9.1. For detailed kinematic analysis, a nail polish imprint was prepared. Clear nail polish was applied to the bottom side of the first pair of true leaves and allowed to dry completely. The abaxial leaf surface was then carefully placed on a piece of double sided clear tap mounted on a microscopic slide. After 15 min, the leaf was carefully peeled off starting from the petiole. The abaxial epidermal imprint was cleared with a few drops of 70% ethanol and then mounted in water for microscopic investigation using a Carl Zeiss light microscope equipped with an AxioCam ICc 5 camera. For each leaf, two pictures were taken at about 75% and 25% of the distance from leaf tip to cover the basipetal gradient that exits during development as previously described (Asl, et al., 2011). The number of epidermal cells (guard cells and pavement cells) per unit of area was calculated from each of the two pictures and the two values were averaged. The total cell number of a leaf is estimated by dividing the total blade area by the averaged cell number per unit area. The blade area was divided by the total cell number to obtain the cell size. For stomatal index calculation, the number of guard cells were divided by the total number of epidermal cells in a unit leaf area as described previously (Nelissen, et al.,

2013). For all cellular analyses, regions near the mid vein and the border of leaves were avoided as recommended (Vanhaeren, et al., 2015).

Quantitative RT–PCR

14 days old seedlings were used for RNA extraction from whole rosette using the RNeasy Plant Mini kit (Qiagen, Germantown, MD). The isolated RNA was treated with DNAse (Ambion Turbo DNA-Free kit) and reverse transcribed with Invitrogen SuperScript III 1st Strand Synthesis System following manufacturer's protocol. *C4H* transcripts were amplified with the primers oXL1169 (5′-CGTCTGAGAATGGCGATTCC-3′) and oXL1170 (5′-CTTCGCATCATGGAGGTTCA-3′). AT1G13320 was used as the reference gene and was amplified with the primers oXL1100 (5′-TAACGTGGCCAAAATGATGC-3′) and oXL1101 (5′-GTTCTCCACAACCGCTTGGT-3′). SYBR-green master mix was used for quantitative PCR reactions (Applied Biosystems, Foster City, CA). The relative gene expression levels were calculated using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008) using a 7500 Fast Real-Time PCR system (Applied Biosystems).

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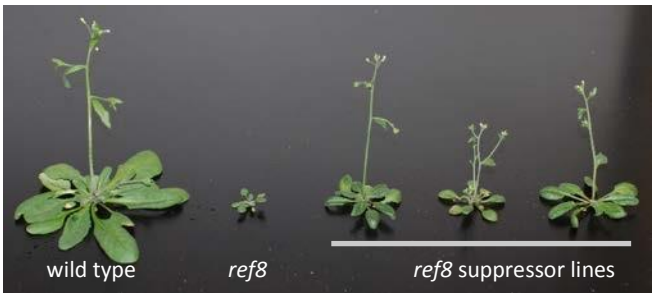

Figure 1. Isolation of *ref8* suppressor lines. Pictures of three week old plants showing the growth alleviation phenotype of three independent suppressor lines.

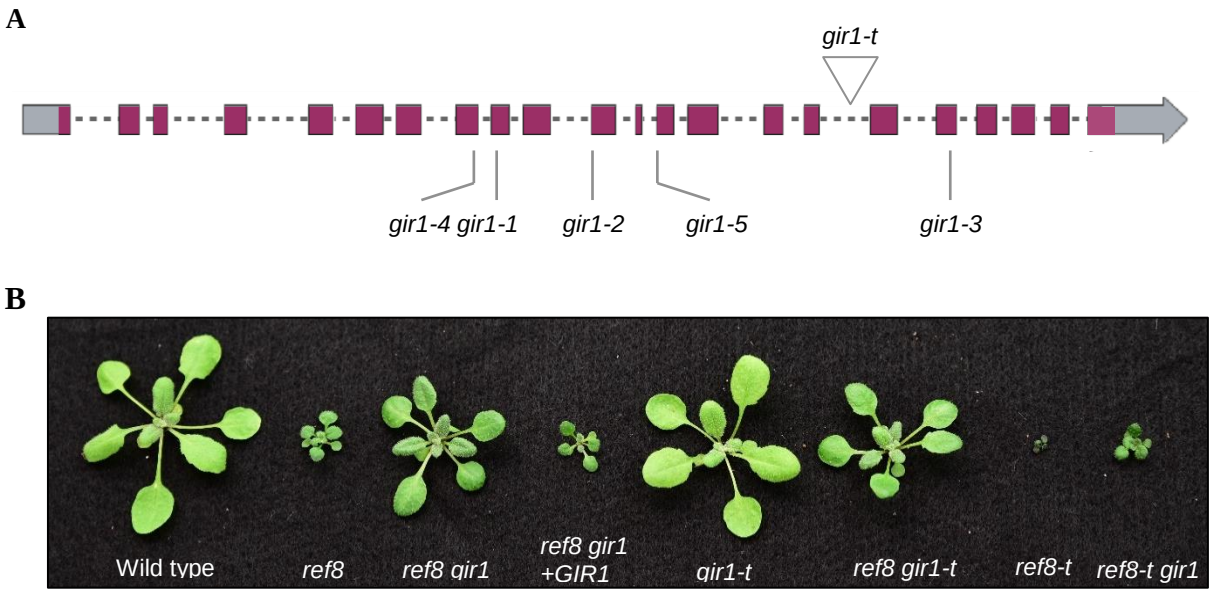

Figure 2. *GIR1* identification and validation.

(A) Schematic drawing of the *GIR1* gene model. Exons are represented by solid maroon boxes, introns by broken lines, and the UTRs by grey boxes. The arrow indicates the direction of transcription. Five alleles were isolated from the *ref8* suppressor screen. The *gir1-1*, *gir1-3*, and *gir1-4* alleles have a premature stop codon; *gir1-2* and *gir1-5* have a mutation at the 3′ end of an intron. The *gir1-t* allele harbors a T-DNA in the 16th intron. **(B)** Validation of the involvement of *GIR1* in *ref8* dwarfism through functional complementation, with a T-DNA allele (*gir1-t*) and with a null allele (*ref8-t*). Shown are the images of three week old wild-type, *ref8*, *ref8 gir1*, *ref8 gir1 + GIR1*, *gir1-t*, *ref8 gir1-t*, *ref8-t* and *ref8-t gir1* rosettes. *ref8 gir1* is significantly bigger than *ref8* and introducing a wild-type copy of the *GIR1* gene back to *ref8 gir1* for functional complementation test eliminated the growth difference. The *ref8 gir1-t* plants are shown to have bigger rosettes. *gir1* mutation also alleviated the growth inhibition phenotype of the *ref8* null mutant (*ref8-t*).

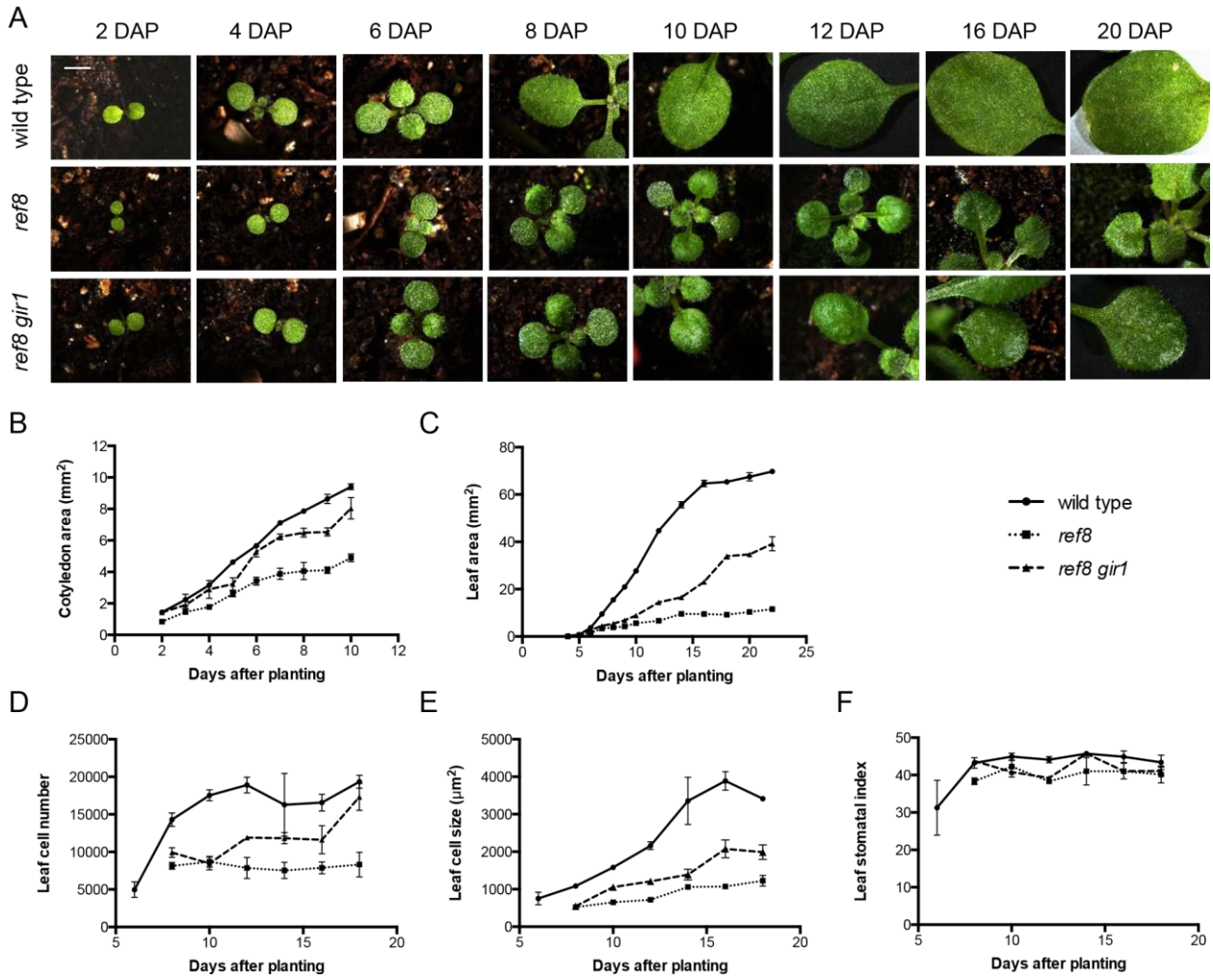

Figure 3: Kinematic analysis of leaf growth in wild type, *ref8* and *ref8 gir1*.

(A) Representative images of cotyledons and first pairs of true leaves at different stages. The seedlings were photographed with a stereoscope from 2 to 20 days after planting (DAP). Only true leaves are shown for wild type at 8 DAP, *ref8* at 16 DAP, and *ref8 gir1* at 10 DAP. Bar = 2 mm. **(B)** Cotyledon growth curve. Total area of cotyledon was plotted from 2 to 10 DAP. **(C)** Leaf growth curve. Total leaf area measurement based on the first pair of leaves from 4 to 22 DAP. **(D)** Total cell number in leaves. Leaf cell number was analyzed every other day from 6 to 18 DAP for wild type and from 8 to 18 DAP for *ref8* and *ref8 gir1*. **(E)** Average cell size in leaves. Leaf cell size was calculated from the same leaves analyzed in D. **(F)** Leaf stomatal index. The percentage of guard cells over total epidermal cells was calculated and plotted**.** In B to F, each data point represents mean of three biological replicates, and the error bars represent standard deviation.

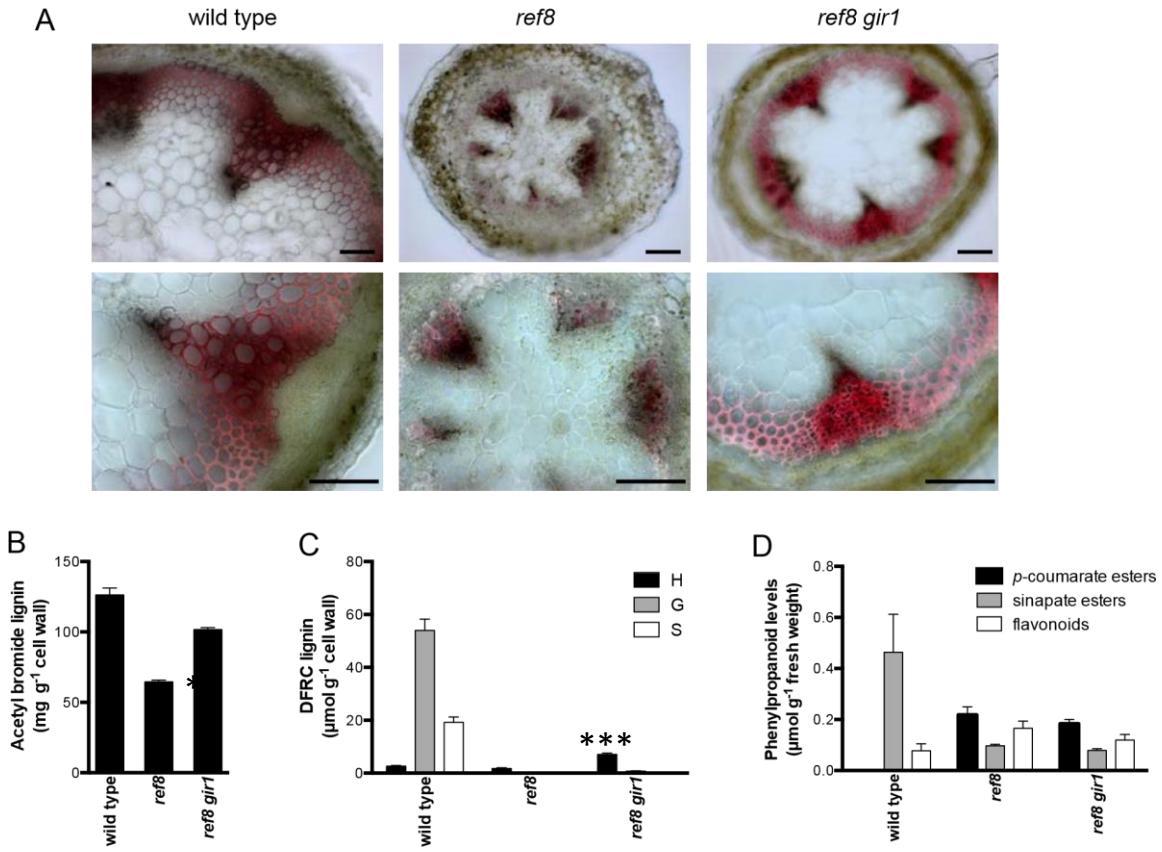

Figure 4. Lignin and soluble phenylpropanoid phenotypes of *ref8 gir1*.

(A) Phloroglucinol staining of stem sections from six week old wild-type, *ref8*, and *ref8 gir1* plants. Bars = 50µm**. (B)** Total lignin content of inflorescence stem from eight week old plants measured by the acetylbromide assay. Double asterisks indicate *P* value < 0.01 using a two tailed Student's *t* test for comparison between wild type and *ref8 gir1*. **(C)** Lignin composition of inflorescence stem from eight week old plants determined by the Derivatization Followed by Reductive Cleavage (DFRC) assay. H, *p*-hydroxyphenol; G, guaiacyl; S, syringyl lignin units. Triple asterisks indicate *P* value <0.001 using a two tailed Student's *t* test for comparison between *ref8* and *ref8 gir1* **(D)** Phenylpropanoid profiles of 14 days old rosettes. In B-D, means of three biological replicates are shown and error bars represent standard deviation.

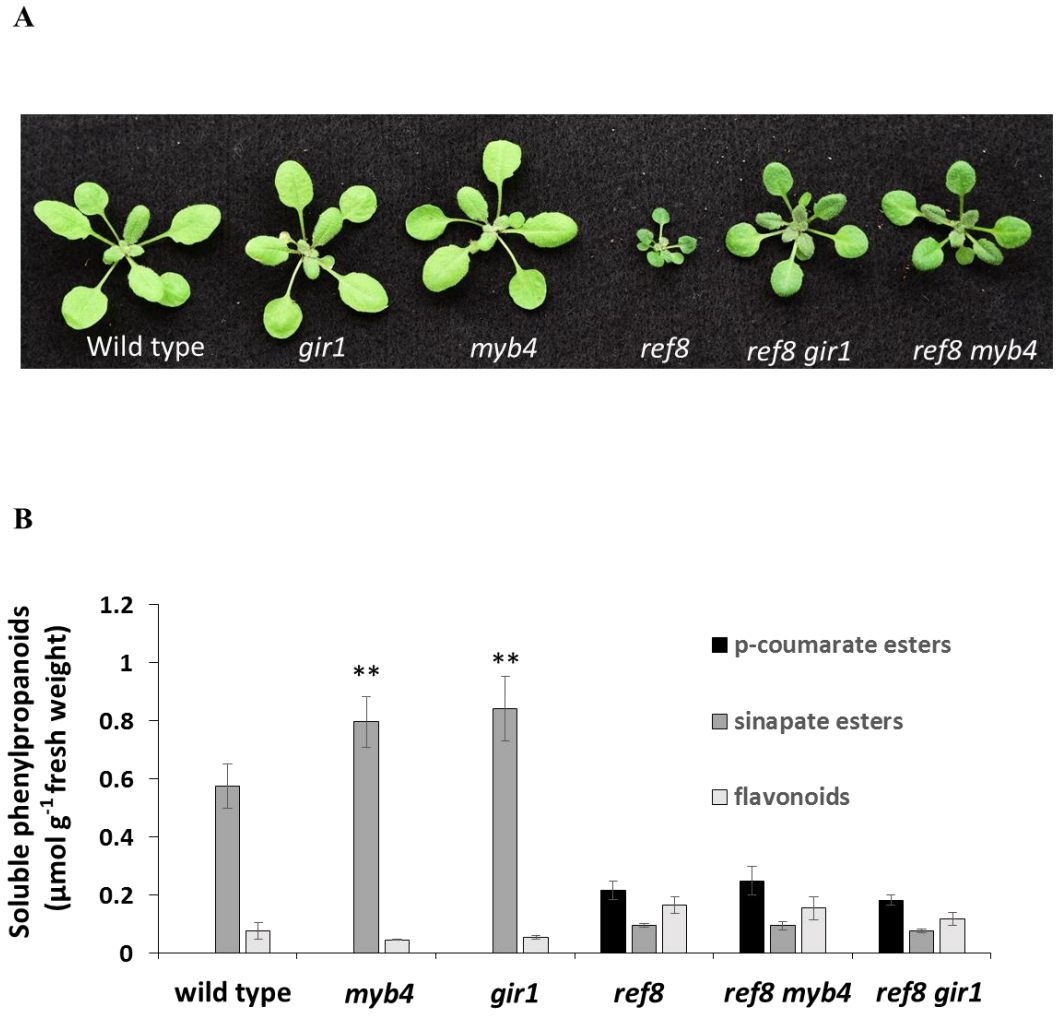

Figure 5. Comparison of *ref8 myb4* and *ref8 gir1* with wild type, *gir1*, *myb4* and *ref8*

(A) The *ref8 myb4* and *ref8 gir1* plants show similar extents of growth alleviation compared with *ref8*. Shown are 3 week old plants. **(B)** The accumulation of *p*-coumarate esters (sum of *p*-coumaroylglucose, *p*-coumaroylshikimate, and *p*coumaroylmalate levels), sinapate esters (sum of sinapoylglucose and sinapoylmalate levels), and flavonoids (sum of kaempferol 3-*O*-[6′ ′ -*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside, kaempferol 3*O*-glucoside 7-*O*-rhamnoside, and kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside levels) in two week old plants. Data represent the mean of at least three biological replicates, and error bars indicate standard deviation. Double asterisks indicate *P* value < 0.01 from a comparison with wild type by a two-tailed Student's *t* test.

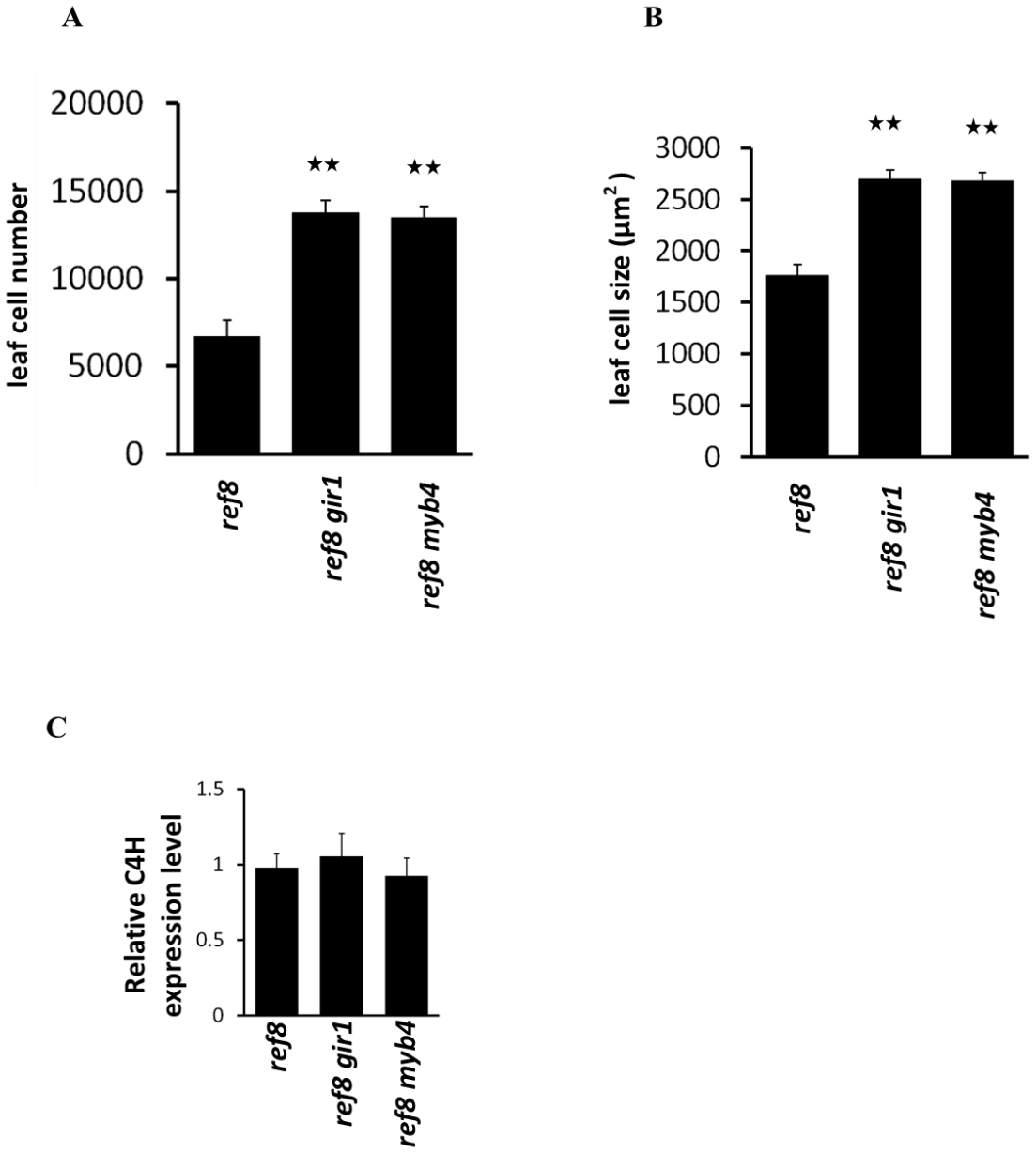

Figure 6. Comparison of *ref8 myb4* and *ref8 gir1* with *ref8*

(A) Leaf cell number analysis was based on the first leaf pair of 18 days old plants.
(B) Leaf cell size analysis done with the same samples used in (A).
(C) Relative expression levels of *C4H* in two week old rosettes determined by qRT-PCR. The accumulation In (A) to (C), data represent the mean of at least three biological replicates, and error bars indicate standard deviation. Double asterisks indicate *P* value < 0.01 from a comparison with *ref8* by a two-tailed Student's *t* test.

Supplementary Materials

Table S1. Primer sequences used in genotyping

Name	Sequence
oXL1001	5′-AATGCGTGGTGAAAGAGTCA-3′
oXL1002	5′-TGCAGGAGTTCAAGGAATCA-3′
oXL1003	5′-ATTTTGCCGATTTCGGAAC-3′
oXL1041	5′-GGTCACTGAATTTTAAGTTCTTGG-3′
oXL1042	5′-GAAAAGTTCCAAGCAGAAGAGC-3′
oXL1034	5′-ATACACCAAGCGTCAAACCTG-3′
oXL1035	5′-CATCACGGTAGCACTTCCAC-3′
oXL1242	5′-GGCTTGAGTTCAAGGCCATA-3′
oXL1241	5′-CGAGTCCAGGGTTTTCAGAC-3′

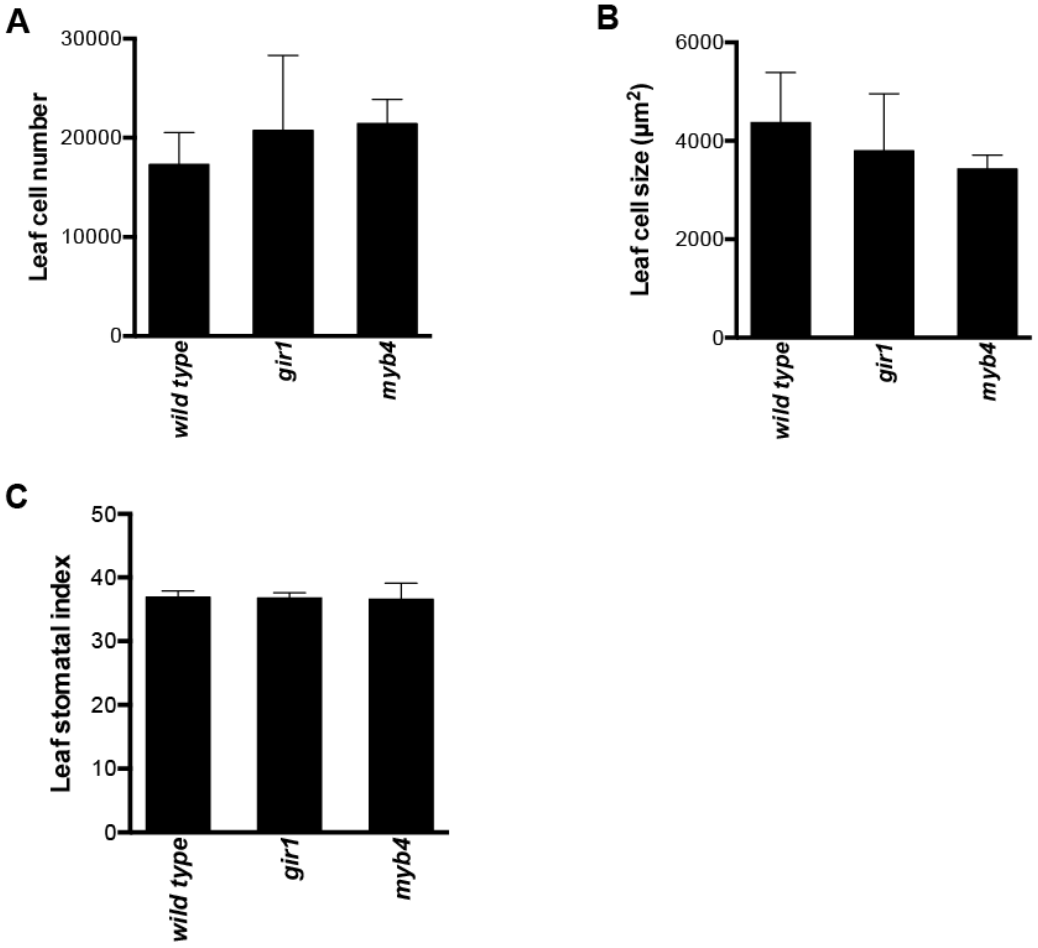

Figure S1. Kinematic measurements of *myb4* and *gir1* mutants.

(A) Leaf cell number measurement based on the first leaf pair on 18 day old plants.

(B) Leaf cell size analysis done with the same samples used in A.

(C) Stomatal index calculated from guard cell density and total epidermal cell density.

In A to C, data represent mean of three biological replicates, and error bars represent standard deviation.

CHAPTER 3

Genetic interactions of *growth inhibition relieved 1* (*gir1*)

ABSTRACT

Investigation of genetic interaction of *gir1* with other suppressors will help us to investigate the LMID pathway. Detailed characterization of the mutant phenotypes will suggest if the interacting partners of *gir1* are likely part of the same pathway or they are positioned on independent pathways. It is also worthwhile to test if the suppressor genes are involved in mediating dwarfism in other low lignin dwarf mutants. Here, we perturbed both *GIR1* and *GIR2* in *ref8* background to generate the *ref8 gir1 gir2* mutant. The triple mutant grows significantly bigger than either of the suppressors alone, suggesting an additive or synergistic interaction between *gir1* and *gir2* in mediating LMID and that *GIR1* and *GIR2* are likely to be on independent pathways. The soluble phenylpropanoid phenotype of *ref8 gir2* double mutants and *ref8 gir1 gir2* triple mutants was not restored, suggesting that the growth restoration is not dependent on upregulation of phenylpropanoid metabolism. Characterization of the cellular phenotype revealed that *gir1* and *gir2* have an additive effect on cell size in *ref8* background, and *gir2* has opposite effect on cell number in wild type and *ref8* mutant background. Furthermore, *gir1* was not able to rescue *ccr1* and *ref3-2* lignin biosynthesis mutants, suggesting that *GIR1* specifically mediates dwarfism in plants defective in *C3'H* gene. However, *gir1* was able to rescue *ref4-3,* a gain of function mutant of REF4, one of the subunit of a large multiprotein transcriptional modulator called Mediator. *ref4-3* also exhibits LMID phenotypes and rescue of its dwarfism by *gir1* suggests the genetic interaction between GIR1 importin and REF4, a negative repressor of phenylpropanoid pathway.

INTRODUCTION

In 1909 Bateson coined the term "epistasis" to describe how one gene masks the effect of another (Bateson, 1909). The phenotypical outcome obtained by generating mutants affecting the same biological process is often indicative of the type of genetic interactions (Mani, et al., 2008; Phillips, 1998). For instance, if the phenotype of a double mutant corresponds to the sum of the phenotypes exhibited by each single mutant, then this type of interaction is called additive (Koornneef, et al., 1998). If this effect is stronger, then this interaction is deemed synergistic. An aggravating interaction results in a more severe phenotype in the double mutant compared to the single mutant and a suppressive interaction as a result of mutation in the second site alleviates the defect arising from the mutation at the first locus (Koornneef, et al., 1998). Over the years, molecular geneticists have utilized epistasis as a tool to dissect genetic pathways (Boucher and Jenna, 2013; Phillips, 2008). Flower color in sweet peas is a classic example of epistasis (Phillips, 1998). In this case, two colorless flowers were crossed to obtain offspring with purple flowers. The F2 segregation ratio of 9:7, suggested that two genes are functionally redundant in the anthocyanin pathway and that a loss of function mutation in either gene can disrupt flower pigmentation. In another pioneering experiment, Beadle and Tatum crossed different knockouts, defective in a particular function to examine the nature of genetic interactions and to order the position of genes in a biochemical pathway (Phillips, 1998). Epistasis analysis of genes involved in substrate dependent pathways like vulval differentiation in *C.elegans*, were also carried out to predict the order of events (Avery and Wasserman, 1992; Huang and Sternberg, 1995).

Depending on the type of pathway, the downstream gene can be epistatic to the upstream gene and vice-versa (Huang and Sternberg, 1995). For instance, a triple response phenotype characterized by an exaggerated apical hook, thick hypocotyl and short root is observed in ethylene related mutants as a part of a regulatory pathway (Guzman and Ecker, 1990). CTR1 is a kinase involved in ethylene signaling and *ctr1* mutants exhibit a constitutive triple response phenotype (Kieber, et al., 1993). EIN2 is required for transduction of ethylene signal and *ein2* mutants don't respond to either endogenous or exogenous ethylene treatment (Alonso, et al., 1999). The *ctr1ein2* double mutant mimicks the *ein2* phenotype suggesting that *EIN2* acts downstream of *CTR1* (Stepanova and Alonso, 2009). In a biosynthetic pathway however, the upstream gene is epistatic as a block at this point will eliminate the formation of any downstream product. A common textbook example involves variation of kernel color in corn (Ford, 2000). The four kernel colors; yellow, red, purple, white are under genetic control of *Pr1*, *C1*, *R1* and *Y1* loci. *Pr1* and *Y1* are structural genes encoding a flavonoid hydroxylase and, a phytoene synthetase, involved in the anthocyanin and carotenoid pathways respectively. Both *C1* and *R1* encode for transcription activators that regulate various structural genes within the anthocyanin pathway. Yellow color of the kernel is due to carotenoids, red and purple are due to anthocyanins and white if both these pigments are absent. A kernel is purple or red in the presence of dominant *R* allele and either yellow or white in presence of *r1* recessive allele. Therefore, *rr* genotype can mask the effect of other genes involved in kernel color to produce yellow kernels. The *C* gene has three major alleles, *C*, *c*, *C'*. The allele *C'* is dominant to both *C* and *c*, and the allele *C* is dominant to *c*. Presence of at least one copy of C is required for

purple kernels, genotype cc gives rise to yellow kernels but the presence of a single copy of C' masks other genes to give a yellow kernel phenotype. So if a hypothetical cross were to be made with one parent having the *RR* and *CC* genotype with another having the *rr* and *CC* genotype, the F2 population will segregate in to three quarter purple and one quarter yellow kernels. Another analysis of epistasis with floral development genes positioned Arabidopsis *LFY* upstream in the floral development pathway (Samipak, 2012). Perturbation of *LFY* leads to abnormal inflorescence structure with bracts but no flowers suggesting it is expressed very early in this pathway. Mutation in *LFY* masks the effects of mutations in downstream components like *AG*, *PI* and *AP2* floral organ homeotic genes involved in specifying the type and arrangement of other floral organs like petals, stamens, sepals and carpels (Busch, et al., 1999). In other words the downstream genes won't be expressed in absence of *LFY* gene product.

Gene-gene interaction studies aided in predicting a functional model that could then be utilized to elucidate functions of the genes influencing trait of interest. Koornneef et al (Koornneef, et al., 1998) generated double mutants to study the genetic interactions among late flowering Arabidopsis mutants. They identified several classes of gene-gene interactions depending on whether they are acting in the same or different developmental pathways. Epistatic genes are assumed to function in the same pathway as observed in the case of late flowering Arabidopsis mutants. If the phenotype is additive or synergistic, it may suggest that the genes are in independent pathways leading to the same effects. For instance, Arabidopsis *GCN5* encoding a histone acetyltransferase has been shown to act in synergy with a receptor

kinase *CLV1*, the interaction of which has been reported to negatively regulate ethylene responses (Poulios and Vlachonasios, 2016). The *gcn5 clv1* double mutant exhibits a strong triple response phenotype, reminiscent of mutants overproducing ethylene (Poulios and Vlachonasios, 2016). Mutation in the transcription factor EIN3, results in *ein3-1* mutant that doesn't exhibit triple response (Chao, et al., 1997). The *ein3-1 clv1* double mutant looks like *ein3-1* and the *ein3-1 gcn5* looks like *gcn5*, suggesting that *CLV1* is upstream and *GCN5* is downstream of *EIN3* respectively. (Poulios and Vlachonasios, 2016).

Here we report the genetic interaction analysis of the two suppressor genes, *GIR1* and *GIR2* that mediate dwarfism in *ref8* mutant. GIR1 as discussed earlier in chapter 2 is a beta importin involved in nuclear localization of proteins and GIR2 is a transcription factor. In order to understand if these two genes function in the same LMID pathway or not, we crossed *gir1 ref8* with *gir2 ref8* to generate *gir1 gir2 ref8* mutant. Comparative analysis of these mutants suggests that *gir1* and *gir2* interact additively and therefore, are likely involved in independent pathways mediating LMID. To test if *GIR1* is necessary for LMID in other lignin biosynthesis mutants, we crossed *gir1* to *ccr1* (Jones, et al., 2001a) and a *C4H* mutant, *ref3-2* (Stout, et al., 2008; Bonawitz, et al., 2012; Schilmiller, et al., 2009), to generate and analyze the double mutants. *gir1* was not able to rescue *ccr1* and *ref3-2* suggesting that different mechanisms may be involved in mediating dwarfism in these mutants. A gain of function mutant of a eukaryotic mediator subunit Med5a/REF4, known as *ref4-3*, also exhibits dwarfism and collapsed xylem (Stout, et al., 2008; Bonawitz, et al., 2012). REF4 is not involved in lignin biosynthesis but is known to modulate phenylpropanoid levels in response to external and internal cues (Stout,

etal., 2008; Bonawitz, etal., 2012). Interestingly, *gir1* was able to rescue *ref4-3*, suggesting that both *GIR1* and *REF4* genetically interact in mediating LMID.

RESULTS

gir1 genetically interacts with *gir2*

GIR1 and *GIR2* were identified in the suppressor screen of Arabidopsis *ref8* mutant. Both *ref8 gir1* and *ref8 gir2* grow significantly bigger than *ref8* plants (Figure 1). GIR1 has been previously characterized to function as an importin protein and GIR2 has been mapped to a locus that encodes a transcription factor. To investigate the genetic interaction between *gir1* and *gir2* in the context of LMID, *ref8 gir1* plants were used to cross pollinate *ref8 gir2*. The F2 progeny were found to have either a *ref8* like, suppressor like, or a larger than suppressor phenotype in a ratio of 60:27:7, which fits the expected segregation ratio of 9:6:1 in case *ref8 gir1 gir2* grows significantly bigger than the double mutants (X^2 test p-value= 0.2). PCR based genotyping confirmed that the growth rescue observed in the "bigger than suppressor" plants is due to the *ref8 gir1 gir2* genotype. The growth phenotype of *ref8 gir1gir2* plants was confirmed in analyzing the subsequent generation. Closer examination revealed that *ref8 gir1 gir2* plants are fertile, set normal siliques and grow bigger than either suppressor alone, suggesting that *GIR1* and *GIR2* interact additively or synergistically in mediating *ref8* dwarfism (Figure 1)**.**

The phenylpropanoid pathway is not upregulated in the plants with *ref8* background

Phenylpropanoid pathway is involved in the synthesis of *p*-coumarate esters, sinapate esters and flavonoids, in addition to lignin. Perturbation of lignin biosynthesis may result in altered soluble metabolite profile in the affected plants. To investigate if the growth rescue observed in the *ref8 gir1, ref8 gir2* suppressor and the *ref8 gir1 gir2* triple mutant is due to the restoration

of phenylpropanoid metabolism, we quantified the abundance of soluble phenylpropanoids using HPLC. *ref8* has a distinct biochemical phenotype with reduced sinapate esters and overproduction of *p*-coumarate esters. It also hyper accumulates stress inducible flavonoids. Our metabolite profiling analysis revealed that the biochemical phenotype of all mutants in *ref8* background is indistinguishable from that of *ref8* (Figure 2). This suggests that *C3'H* is still blocked in the mutants and the growth rescue may not be dependent on the upregulation of the phenylpropanoid pathway

Kinematic analysis of cotyledon and leaf growth in *ref8*, *ref8 gir1*, *ref8 gir2*, *ref8 gir1 gir2* and wild-type plants

In chapter 2, we have demonstrated that *ref8* is defective in both cell proliferation and cell expansion. We have also shown that *gir1* and *myb4* had similar effects on alleviating the *ref8* cellular defects. This observation together with the knowledge of MYB4 as a cargo of GIR1 (Zhao, et al., 2007) suggests that *GIR1* and *MYB4* may be acting on the same step of the LMID pathway. Mutation in GIR2, a transcription factor also rescues *ref8* dwarfism but to a lesser degree relative to *gir1*. As described above, disruption of both *GIR1* and *GIR2* results in better rescue of *ref8* dwarfism than either *gir1* or *gir2* alone. To further characterize the effect of *gir1* and *gir2* on *ref8* growth, we tracked the growth of true leaf and cotyledon over time. Consistent with the whole plant phenotype, our kinematics data indicate that *gir1* and *gir2* interact additively in regulating leaf growth in the *ref8* background (Figure3). The *gir1* mutation had a bigger effect on increasing the size of cotyledons and leaves in *ref8* background than *gir2* (Figure 3C and 3D). Interestingly, simultaneous disruption of both *GIR1* and *GIR2*

had similar effects as *gir1* alone on alleviation of cotyledon growth inhibition in *ref8*, suggesting that GIR1 may be epistatic to GIR2 in regulating cotyledon size (Figure 3A and 3C). Analysis of the true leaf growth pattern in the mutants revealed an additive interaction between *gir1* and *gir2*, as the leaf size of *ref8 gir1 gir2* mutant is significantly bigger than either suppressor alone (Figure 3D). Closer examination of the phenotype also revealed that the *ref8 gir1 gir2* plants are already bigger than both *ref8 gir1* and *ref8 gir2* suppressors as early as 4 days post germination (Figure 3A).

***ref8 gir1 gir2* plants have similar cell number as *ref8 gir1* plants**

Previously in chapter 2, we have discussed the cellular phenotype of both *ref8* and *ref8 gir1* plants in detail. *ref8* has fewer cells. Mutation in *GIR1* leads to a dramatic rescue of cell number in *ref8 gir1* plants (Figure 4). The *GIR2* knockout has fewer cells relative to wild-type plants (Figure 4). Mutation in *GIR2* also leads to rescue of *ref8 gir2* cell number, suggesting an opposite effect of *gir2* in wild type and *ref8* background (Figure 4). However, mutation in both *GIR1* and *GIR2* leads to rescue of cell number similar to *ref8 gir1*, suggesting *GIR1* may be epistatic to *GIR2* and *GIR2* may be epistatic to *REF8* in cell division process (Figure 4).

***gir1* and *gir2* have additive effect on the cell size of *ref8 gir1 gir2* mutants**

ref8 has smaller cells and mutation in *GIR1* leads to rescue of cell size in *ref8 gir1* was shown in chapter 2. Here, our data suggest that mutation in *GIR2* also leads to rescue of cell size in *ref8 gir2* mutants (Figure 5). Interestingly, mutation in both *GIR1* and *GIR2* leads to rescue of cell size to a greater degree in *ref8 gir1 gir2* mutants, suggesting an additive interaction between *gir1* and *gir2* in regulating cell expansion in these mutants (Figure 5). The

bigger cell size observed in the *GIR2* knockout mutant suggests that *GIR2* may be involved in negatively regulating cell size. However, we cannot exclude the possibility that this increase in cell size could be a compensatory effect (Mizukami and Fischer, 2000; Narita, et al., 2004; Horiguchi, et al., 2006; Anastasiou, et al., 2007; Tsuge, et al., 1996; Kim, et al., 2002; Fujikura, et al., 2007), as a result of reduced cell number due to the *GIR2* mutation (Figure 4).

gir1 has no effect in _ccr1_ and _ref3-2_ background but can rescue _ref4-3_ mutants

Both *CCR1* and *C4H* as discussed in chapter 1 are lignin biosynthetic genes. *ccr1* mutants are dwarf with smaller but significantly more cells relative to the wild-type plants (Xue, et al., 2015). *ccr1* mutants also exhibit a collapsed xylem phenotype, hypothesized to result from impaired water transport due to reduced lignin (Jones, et al., 2001b). To test if mutation in *GIR1* can also rescue *ccr1* dwarfism, *gir1* plants were crossed with *ccr1* plants. The F2 progeny with *gir1/ gir1 ccr1*/*CCR1* genotype were allowed to self to isolate the double mutants in the subsequent generation. The F3 population segregated into two phenotypes, *ccr1*-like and wild-type-looking plants with a ratio of ~1:3 (33 *ccr1* looking and 96 wild type looking). PCR based genotyping of both *GIR1* and *CCR* genes was used to identify the double mutant homozygous for both *ccr1* and *gir1*. We compared the growth of *ccr1* and *ccr1 gir1* side by side which suggested no growth rescue (Figure 6A). The *ref3-2* mutant is defective in *C4H* gene and also exhibits LMID phenotypes (Schilmiller etal. 2009). The *gir1 ref3-2* double mutant was isolated in F3 generation using a parent with *gir1/ GIR1 ref3-2*/*ref3-2* genotype. The *ref3-2* and *gir1 ref3-2* double mutants were genotyped and further analyzed side-by-side with no growth rescue observed (Figure 6B). As, *gir1* was not able to rescue *ccr1* and *ref3-2*

dwarfism, it is possible that different mechanisms may be involved in mediating dwarfism in these mutants.

REF4/MED5a is a component of eukaryotic Mediator, a multiprotein complex that acts as a bridge between basal transcription machinery at the core promoter and transcriptional activators during RNA polymerase II mediated transcription (Björklund and Gustafsson, 2004; Bonawitz, et al., 2012). REF4 is involved in regulating lignification and mutation in the Gly-383 residue of Arabidopsis REF4, leads to dominant mutant *ref4-3* which exhibits dwarfism and decreased accumulation of all phenylpropanoids including lignin compared to the wild-type plants (Stout, et al., 2008; Bonawitz, et al., 2012; Bonawitz, et al., 2014a). In order to test if mutation in *GIR1* could rescue *ref4-3* dwarfism, we generated and analyzed *ref4-3 gir1* double mutants. The F2 population was used to identify plants with *ref4-3/REF4 gir1/gir1* genotype. PCR based genotyping was used to identify the *ref4-3 gir1* double mutants in the F3 population. The *ref4-3 gir1* plants look bigger than *ref4-3*/*ref4-3* homozygous plants when grown side by side (Figure 7).

DISCUSSION

Severely lignin-depleted plants exhibit growth and developmental anomalies called Lignin Modification Induced Dwarfism (LMID). Notably, vascular collapse is widely observed in response to alteration of lignin biosynthetic genes early in the lignin pathway (Chen and Dixon, 2007; Li, et al., 2008b; Bonawitz and Chapple, 2010; Bonawitz and Chapple, 2013). This defect is commonly attributed to the biological role of lignin as a structural component of the secondary cell wall that confers mechanical strength and help avoid cavitation during drought. For instance, the effects of LMID in *cse* and *c4h* low lignin mutants was recently shown to be lessened if not completely eliminated by expressing the lignin biosynthetic genes Caffeoyl Shikimate Esterase (*CSE*) and Cinnamate 4-hydroxylase (*C4H*) under vessel specific promoters of transcription factors *VND6* and *VND7* respectively (Vargas, et al., 2016; Yamaguchi, et al., 2010b). Water stress arising from defective water transport may be a common cause leading to dwarfism in most lignin deficient mutants. We sought to investigate whether loss of *GIR1* function has a similar growth effect on other lignin mutants with a xylem vessel collapse phenotype, such as *ccr1* and *ref3-2. gir1* wasn't able to rescue *ref3-2* and *ccr1* mutants, suggesting that the dwarfism in these mutants might be due to different mechanisms.

Previously in chapter two, we showed that the phenylpropanoid profile is not rescued in the *ref8 gir1* suppressor. Here, we extended the metabolite analysis to include another independent suppressor *ref8 gir2* and a triple mutant *ref8 gir1 gir2*. Similar to the *ref8 gir1* plants, the soluble metabolite phenotype of *ref8 gir2* and *ref8 gir1 gir2* plants was not rescued,

suggesting that the growth rescue is independent of phenylpropanoid metabolism and that the block is still there in the *C3'H* step of the lignin biosynthesis pathway.

A possible connection between lignin pathway perturbation and changes in cellular processes was recently suggested by an Arabidopsis *ccr1* mutant (Xue, et al., 2015). Disruption of *CCR1* leads to hyper-accumulation of ferulic acid that causes dwarfing in *ccr1* plants. They also proposed that the defect in the lignin biosynthetic *CCR* gene results in delayed exit from cell proliferation during leaf development, which results in more but smaller cells relative to wild type (Xue, et al., 2015). Their study highlights the necessity to explore the possibility of cellular defects leading to dwarfism in other lignin mutants including *ref8*. In chapter 2, we showed that *ref8* is defective in both cell division and cell expansion and this defect is rescued in the *ref8 gir1* suppressor plants. In order to score the extent of growth rescue in *ref8 gir2* plants due to mutation in *GIR2*, we closely monitored both cotyledon and first true leaf growth in the *ref8 gir2* mutant (Figure 3A and 3B). Consistent with the overall growth phenotype of *ref8 gir2* plants, our analysis suggests that both leaf and cotyledon growth is significantly rescued in the *ref8 gir2* suppressor plants but to a lesser degree compared to *ref8 gir1* suppressor plants (Figure 3C and 3D). Interestingly, when both *GIR1* and *GIR2* are mutated in *ref8* background, growth is dramatically rescued in *ref8 gir1 gir2* plants to a much greater extent compared to either suppressor alone (Figure 3). This suggests an additive effect of *gir1* and *gir2* on *ref8* growth and that *GIR1* and *GIR2* are likely to be on independent pathways.

Our kinematics analysis of the first true leaf pair revealed that *gir2* has significantly less but bigger cells compared to the wild type control (Figure 4 and 5). This is reminiscent of the

compensation syndrome, where by any defect in cell division is compensated by increase in the cell size to attain the final organ size (Mizukami and Fischer, 2000; Narita, et al., 2004; Horiguchi, et al., 2006; Anastasiou, et al., 2007; Tsuge, et al., 1996; Kim, et al., 2002; Fujikura, et al., 2007). Our data revealed that *ref8 gir1 gir2* plants have significantly bigger cells compared to either *ref8 gir1* or *ref8 gir2* plants (Figure 5). Therefore, *gir1* and *gir2* appears to have an additive effect on cell size in *ref8 gir1 gir2* plants. Interestingly, the number of cells in *ref8 gir1 gir2* was similar to *ref8 gir1*. This suggests that *gir1* is epistatic to *gir2* and *gir2* is epistatic to *ref8* in cell division pathway. While *gir2* has fewer cells, more cell number was quantified for *ref8 gir2*, suggesting an opposite effect of *gir2* mutation in wild-type and *ref8* background.

The mutation of Mediator (a transcriptional coregulatory protein complex) subunits MED5a (REF4) and MED5b (RFR1) in *ref8* mutant background was able to recue its growth to near wild-type level (Bonawitz, et al., 2014a). LMID is genetically controlled process and thereby necessitated the identification of genes and a pathway involved in LMID to eventually enable maximal lignin modification in plants with normal growth, uncompromised vascular integrity and disease resistance. Our analysis also suggests that the LMID pathways involving GIR1 and GIR2 are distinct from the one that involves mediator (Bonawitz, et al., 2014a) because mutations in the mediator subunits results in upregulation of phenylpropanoid pathway (accumulation of soluble metabolites), which is not the case with mutation in either GIR1, GIR2 or both genes in the *ref8* mutant background.

It remains a question as to how plants perceive the changes induced by alteration of lignin biosynthesis and integrate this information into series of downstream events that ultimately leads to stunted growth. According to our genetic interaction data, both GIR1 (through MYB4) and GIR2 may act through independent pathways that ultimately repress cell proliferation and expansion in *ref8* background. It is currently unclear if stunted growth may be an adaptive plant response elicited by plethora of factors including stress (drought/activation of cell wall integrity pathway) and/or altered hormone response and signaling upon perturbation of phenylpropanoid pathway. As GIR1 and GIR2 clearly have profound regulatory effects in LMID pathway, identification of their downstream target genes in *ref8* background offers exciting possibility of elucidating the molecular mechanisms involved in mediating lignin modification induced dwarfism. Alternatively, growth rescue may be through the activation of H-lignin pathway. We have shown in chapter 2 that mutation in GIR1 leads to deposition of more H-lignin in the *ref8 gir1* suppressor plants. Increased deposition of H-lignin is also reported in plants with mutation in the Mediator subunits (*REF 5a/5b*) in *ref8* background (Bonawitz, et al., 2014b). This suggests that as the pathway is still blocked at the *C3'H* step, the flux may be directed to the production of H-lignin, which ultimately results in more total lignin responsible for better growth. As *gir1* was able to rescue *ref4-3* but not *ccr1 and ref3-2* (Figure 6C), characterization of the lignin phenotype in the *ref4-3 gir1*, *ccr1 gir1*, and *ref3-2 gir1* double mutants may provide useful information to understand the link between lignin and growth in the suppressors. Considering the role of REF4 as a transcriptional modulator

(Bonawitz etal., 2014), it is also worth investigating if GIR1 is a component of this signaling pathway that limits growth upon lignin pathway perturbation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Scotts Osmocote Plus slow release fertilizer (Hummert International, Earth City, MO) was added to Redi-earth Plug and Seedling Mix (Sun Gro Horticulture, Agawam, MA) for growing Arabidopsis thaliana plants in a growth chamber. A 16-h light/8-h dark photoperiod was used with a 100 μE m–2 sec–1 light intensity at 21°C. The Arabidopsis *ref8-1* and *ref8*^{popOn} lines have been described previously (Kim, et al., 2014; Franke, et al., 2002b). The T-DNA insertional mutants *gir1* (SALK_133577c) and *gir2* (SALK_059568c) were obtained from the Arabidopsis Biological Resource Center. All the mutants are in the Col-0 background. Both *ref8gir1* and *ref8gir2* were obtained from suppressor screening as described previously in chapter 2. The *ref8gir1gir2* triple mutants were obtained in F2 generation of a cross between homozygous *ref8gir1* and *ref8gir2*. The *ccr1, ref3-2* and *ref4-3* mutant seeds were obtained from Dr. Clint Chapple. The mutant phenotypes were described previously (Stout, et al., 2008; Ruegger and Chapple, 2001; Schilmiller etal. 2009, Bonawitz, et al., 2012; Jones, et al., 2001b; Xue, et al., 2015).

Genotyping

Primer sequences used for genotyping are shown in Table 1. The primers oXL1001 and oXL1002 were used in combination with *Eco*RV digestion for genotyping the endogenous *REF8* locus, as previously described (Kim, et al., 2014). The wild-type allele gives rise to a 865-bp PCR product that is resistant to *Eco*RV digestion, whereas the amplicon from the *ref8-1* allele is cleaved into two fragments (584 bp and 281 bp). Similarly, oXL1041 and oXL1042

were used with Hpy166II to detect the *gir1-1* allele (wild type: 488 bp; *gir1-1*: 357 bp and 131 bp). For T-DNA mutants, the wild-type allele is detected by PCR using a pair of gene-specific primers, LP and RP; and the mutant allele is detected by amplification with a T-DNA border primer (BP, oXL1003) *(Alonso, et al., 2003).* Primers oXL1034 (LP) and oXL1035 (RP) were used for genotyping *gir1*. Primers oXL1250 (forward primer) and oXL1251 (RP) were used for amplifying *gir2*. The amplified PCR product was cleaved by digesting with Bsu361 (mutant = 137bp and 28 bp). Primers oXL1338 (BP) and oXL1336 (RP) were used for amplifying the T-DNA within CCR gene and primers oXL1337 (LP) and oXL1336 (RP) were used for gene specific amplification. The primers oXL1252 and oXL1253 were used in combination with *AVA*II digestion for genotyping endogenous *REF4* locus as described previously (Stout, et al., 2008).

Soluble metabolite profiling

Rosette leaves were harvested and extracted with 50% (v/v) methanol at 60°C for 30 min. Samples are centrifuged at 14,000 g for 5 min before loading on HPLC. Five microliters of extract was separated on an Agilent Eclipse plus C18 column (3- × 100-mm with 1.8 µM particles) using 0.1% (v/v) formic acid in water as solvent A and 0.1% (v/v) formic acid in acetonitrile as solvent B. The soluble phenylpropanoids were separated by a gradient over 28 min, starting with a hold at 2% solvent B for 1 min, then a gradient increase to 20% B over 20 min, and then to 90% B over 1 min and hold at 90% B for 2 min. Data were recorded at 330 nm with a spectral bandwidth of 4 nm. Peak identification and quantification was done as described previously (Kim, et al., 2014).

Kinematics analysis

The whole leaf images were taken using a Zeiss Stereoscope Discovery V20 mounted with PlanApo S 1.0x FWD 60 mm lens. The total area of the leaf was calculated using the curve spline feature under the area measurement tab in the software AxioVision SE64 Rel. 4.9.1. For detailed kinematic analysis, a nail polish imprint was prepared. Clear nail polish was applied to the bottom side of the first pair of true leaves and allowed to dry completely. The abaxial leaf surface was then carefully placed on a piece of double sided clear tap mounted on a microscopic slide. After 15 min, the leaf was carefully peeled off starting from the petiole. The abaxial epidermal imprint was cleared with a few drops of 70% ethanol and then mounted in water for microscopic investigation using a Carl Zeiss light microscope equipped with an AxioCam ICc 5 camera. For each leaf, two pictures were taken at about 75% and 25% of the distance from leaf tip to cover the basipetal gradient that exits during development as previously described (Asl, et al., 2011). The number of epidermal cells (guard cells and pavement cells) per unit of area was calculated from each of the two pictures and the two values were averaged. The total cell number of a leaf is estimated by dividing the total blade area by the averaged cell number per unit area. The blade area was divided by the total cell number to obtain the cell size. The stomatal index was calculated by dividing the number of guard cell by the total number of epidermal cell in unit leaf area as described previously (Nelissen, et al., 2013). For all cellular analyses, regions near the mid vein and the border of leaves were avoided as recommended (Vanhaeren, et al., 2015).

Table 1: List of primer sequences

Name	Sequence
oXL1001	5′-AATGCGTGGTGAAAGAGTCA-3′
oXL1002	5′-TGCAGGAGTTCAAGGAATCA-3′
oXL1003	5′-ATTTTGCCGATTTCGGAAC-3′
oXL1041	5′-GGTCACTGAATTTTAAGTTCTTGG-3′
oXL1042	5′-GAAAAGTTCCAAGCAGAAGAGC-3′
oXL1034	5′-ATACACCAAGCGTCAAACCTG-3′
oXL1035	5′-CATCACGGTAGCACTTCCAC-3′
oXL1250	5′-GCGTAGATGTTCTGGAACTGAAAATACCTT-3′
oXL1251	5′-AAGTTCCCAGCTGTTTTTGATAACTA-3′
oXL1336	5′-GTGTCGTAGAGGCTTTGCTTG-3′
oXL1337	5′-TTG TGG AAA TAT TTC CGG TTG-3′
oXL1338	5′-ATTTTGCCGATTTCGGAA C-3′
oXL1252	5′-CTTTGGTTGCCCATTGATCT-3′
oXL1253	5′-GATTGGTTCCCCCAATTACA-3′

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Figure 1. Phenotype of *ref8 gir1 gir2* triple mutant. Pictures of 4 week old plants showing *ref8 gir1 gir2* plants grow bigger than *ref8 gir1* and *ref8 gir2* suppressor plants alone.

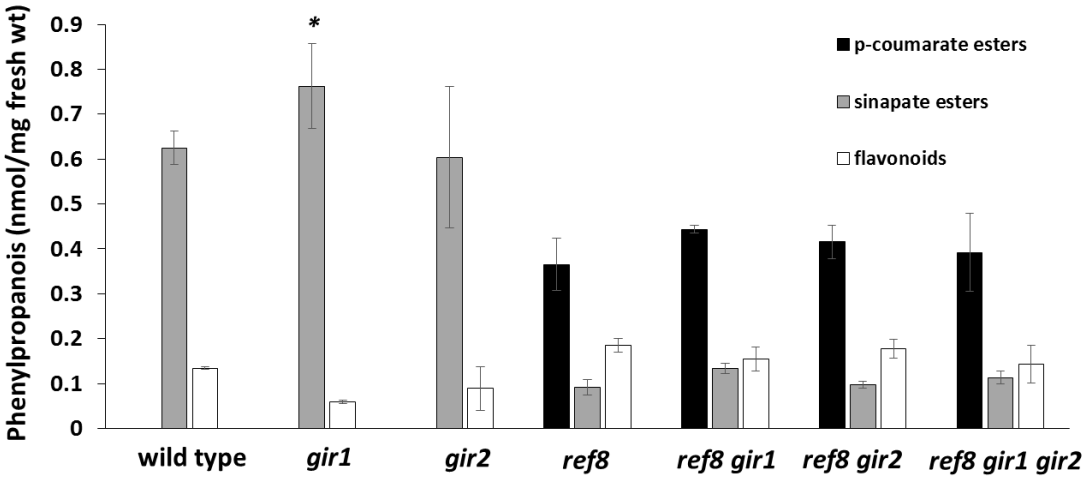

Figure 2. Soluble phenylpropanoid profile of *ref8 gir2* and *ref8 gir1 gir2*
Phenylpropanoid profiles of 14 day old rosettes. Means of three biological replicates are represented, and error bars represent standard deviation. Single asterisk indicates *P* value <0.05 using a two tailed Student's *t* test for comparison between *gir1* and wild type.

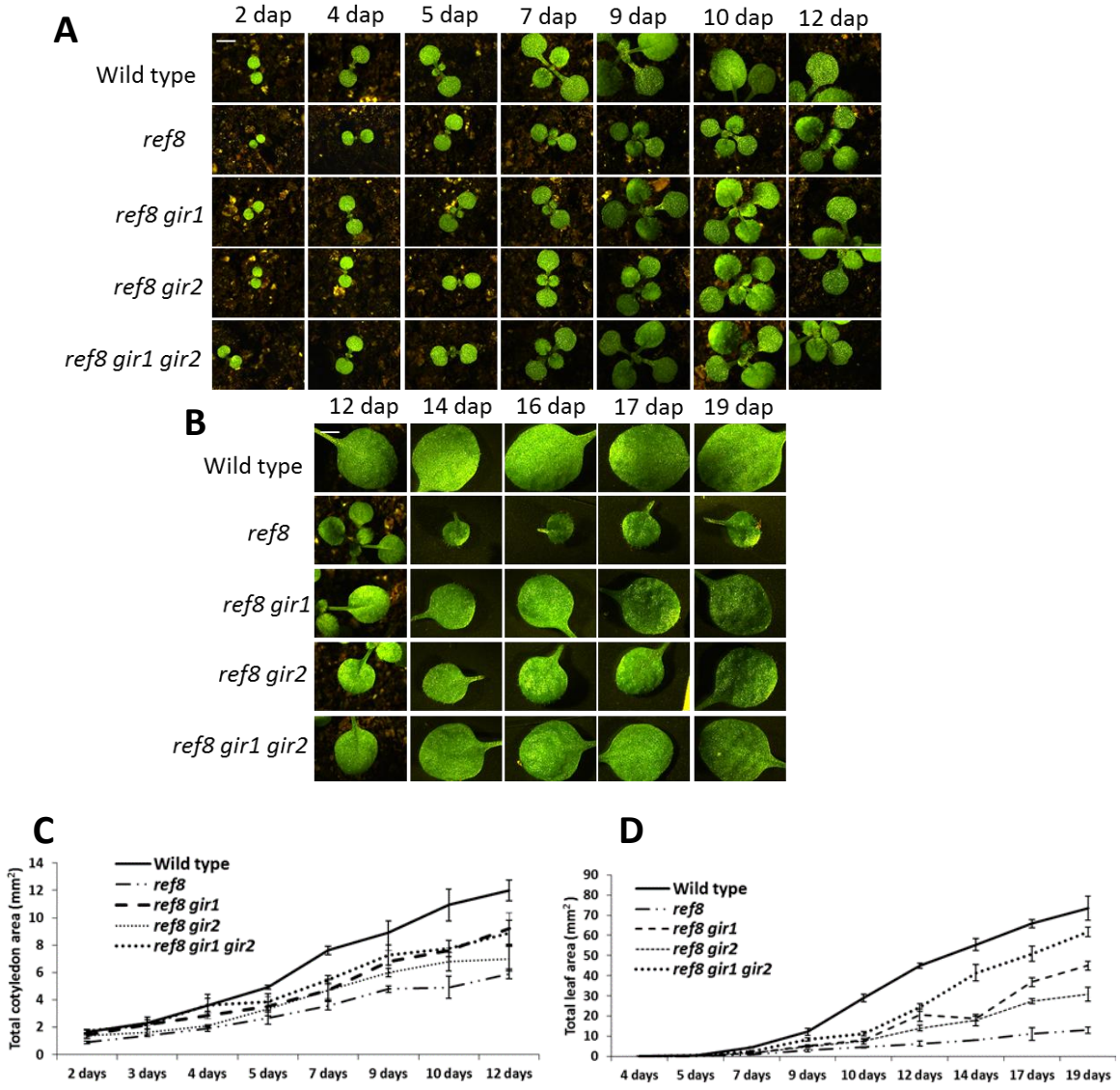

Figure 3: Kinematic analysis of cotyledon and leaf growth in wild type, *ref8*, *ref8 gir1*, *ref8 gir2* and *ref8 gir1 gir2*

(A) and (B) Representative images of cotyledons and the first pair of true leaves at different stages. Seedlings were photographed through a stereoscope 2 to 19 days after planting (DAP). Cotyledons are shown till 12 days. True leaves are shown from 4 days through 19 days. Bar = 2 mm. **(C)** Cotyledon growth curve. Total area of cotyledon was plotted from 2 to 12 DAP **(D)** True leaf growth curve. Total leaf area was measured from 4 to 19 days. Means of three biological replicates are represented. Error bars indicate standard deviation.

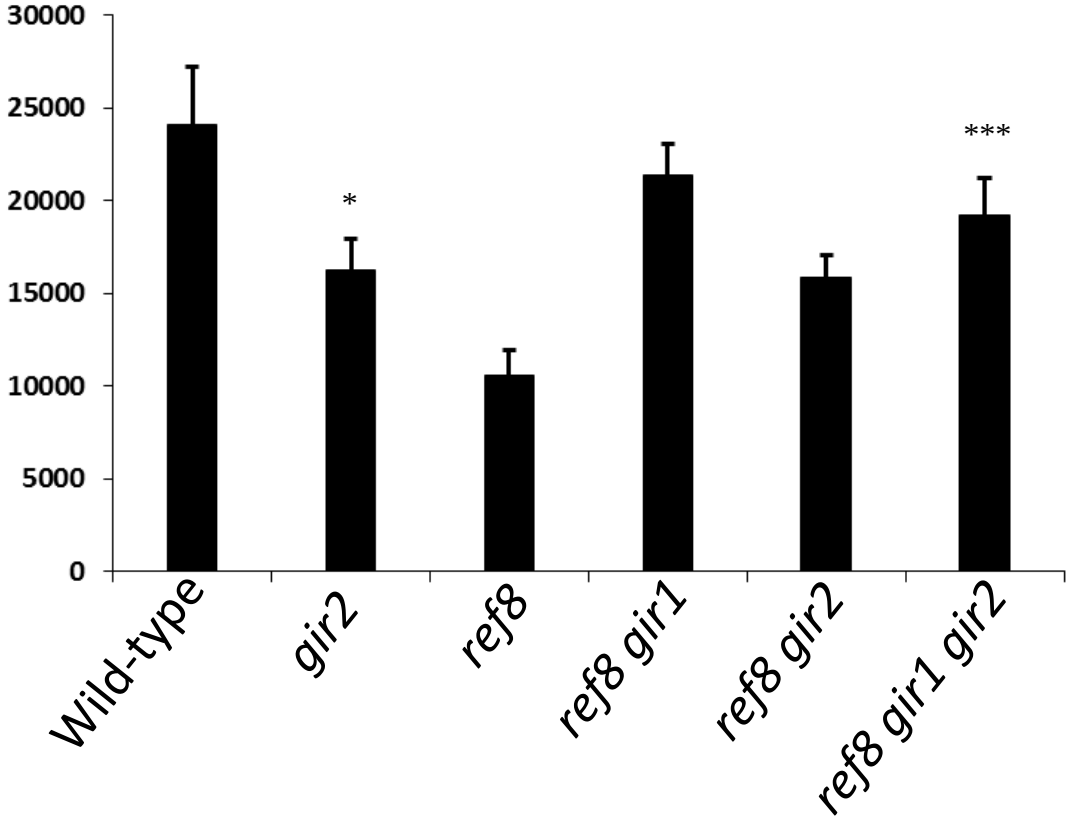

Figure 4: Total cell number in leaves. Leaf cell number in the abaxial leaf epidermis was analyzed at 19 DAP. Means of three biological replicates are represented. Error bars represent standard deviation. Single asterisk indicates *P* value <0.05 using a two tailed Student's *t* test for comparison between *gir2* and wild type. Triple asterisks indicates *P* value <0.001 using a two tailed Student's *t* test for comparison between *ref8 gir1gir2* and *ref8*.

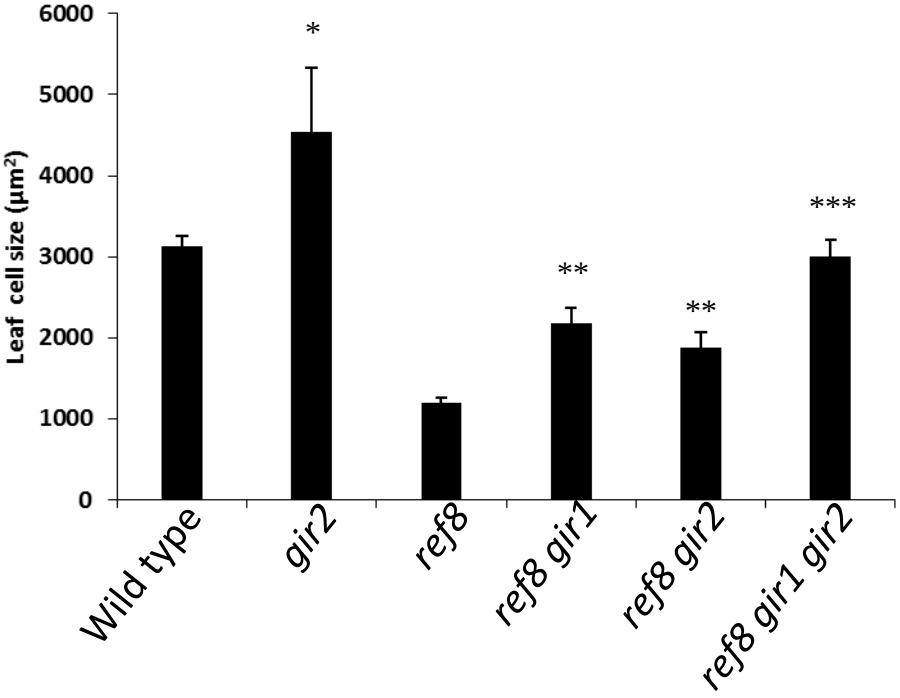

Figure 5: Average cell size in leaves. Leaf cell size was analyzed using the same sample from figure 4. Means of three biological replicates are represented. Error bars represent standard deviation. Single asterisk indicates *P* value <0.05 using a two tailed Student's *t* test for comparison between *gir2* and wild type. Double asterisks indicates *P* value <0.01 using a two tailed Student's *t* test for comparison between *ref8 gir1* and *ref8* and *ref8 gir2* and *ref8*. Triple asterisks indicates *P* value <0.001 using a two tailed Student's *t* test for comparison between *ref8 gir1gir2* and *ref8*.

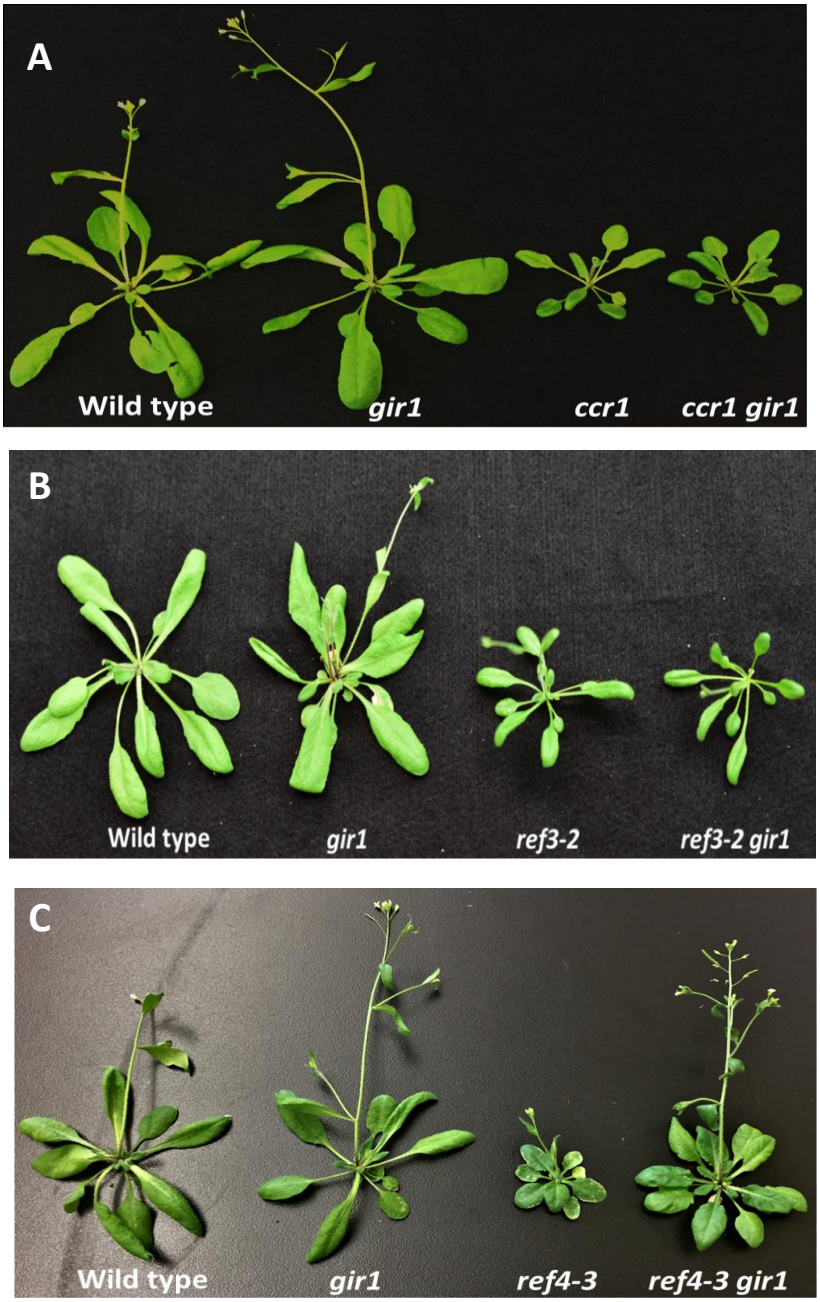

Figure 6: **A**. Growth phenotype of 3.5 week old *ccr1 gir1* double mutant
B. Growth phenotype of 3 week old *ref3-2 gir1* double mutant
C. Growth phenotype of 3 week old *ref4-3 gir1 double* mutant