# The Role of the FEI Receptor Kinases in the Regulation of Cell Wall Function in *Arabidopsis thaliana*

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## ABSTRACT

BLAIRE JANELL STEINWAND: The Role of the FEI Receptor Kinases in the Regulation of Cell Wall Function in *Arabidopsis thaliana*

(Under the direction of Joseph Kieber)

The plant cell wall is a staple in the human diet and provides the raw material used to manufacture paper, textiles, and more recently, biofuel as it is the most abundant reservoir of carbon in nature. In plants, the cell wall provides structural support, acts as a barrier to pathogen attack, and determines both the direction and the extent of cell expansion. The cell wall is a dynamic structure that functions throughout plant growth and development and in response to developmental and environmental cues. Despite the importance of the cell wall, the molecular components and signal transduction pathways involved in regulating its function remain largely unknown.

FEI1 and FEI2 are two leucine-rich repeat receptor-like kinases (LRR-RLKs) that promote cell wall function in *Arabidopsis thaliana*. Mutations in both FEI1 and FEI2 disrupt cell wall synthesis and this leads to a loss of cell elongation and a short, swollen root phenotype. In order to determine how exactly the FEI proteins regulate cell wall function, we sought to identify novel components of the FEI pathway and have isolated suppressors of the *fei1 fei2* mutant phenotype. Further characterization of these as well as known components of the pathway suggests the

FEI proteins may act in a complex with other LRR-RLKs and ACC synthase to post-translationally regulate cell wall synthesis.

*This dissertation is dedicated to my ever-supportive friends and family.*

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| | |
|---|---|
| 2,4D | dichlorophenoxyacetic acid |
| ACC | 1 aminocyclopropane-1-carboxylate |
| ACS | 1 AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE |
| AIB | $\alpha$-aminoisobutyric acid |
| AOA | Aminooxy-acetic acid |
| BAK1 | BRI1 ASSOCIATED KINASE 1 |
| BiFC | Bimolecular fluorescence complementation |
| BRI | BRASSINOSTEROID 1 |
| CESA | Cellulose synthase |
| $Ch_D$ | Discordant chastity |
| COB | COBRA |
| CSC | Cellulose synthase complex |
| DCB | 2,6-dichlorobenzonitrile |
| ELI1 | ECTOPIC LIGNIFICATION 1 |
| FER | FERONIA |
| FIL | FEI INTERACTING LEUCINE RICH REPEAT RECEPTOR LIKE KINASE |
| HERK | HERCULES |
| IXB | isoxaben |
| KOR | KORRIGAN |
| LRR | leucine-rich repeat |

| | |
|---|---|
| MAPK | MITOGEN ACTIVATED PROTEIN KINASE |
| PRC | PROCUSTE |
| RLK | receptor-like kinase |
| RSW1 | ROOT SWELLING 1 |
| SNP | Single nucleotide polymorphism |
| SOS5 | SALT OVERLY SENSITIVE 5 |
| THE | THESEUS |
| WAK | WAL-ASSOCIATED KINASE |
| YFP | Yellow fluorescent protein |

# CHAPTER 1

# BACKGROUND AND SIGNIFICANCE

The plant cell wall is a rigid but highly dynamic structure that provides mechanical support, protection against pathogen attack, and determines the direction and extent of cell expansion (Humphrey et al., 2007). The dynamic nature of the plant cell wall allows growing cells to expand while providing the mechanical strength required to resist the forces of turgor pressure exerted on the cell (Cosgrove, 2000). The properties of the cell wall are modified during growth and development as well as in response to a wide variety of environmental stimuli. In order to maintain the integrity of the wall and to adjust its properties to accommodate the changing needs of the cell, plants respond to perturbations to the wall and environmental cues by remodeling matrix polysaccharides and by regulating the cell wall biosynthetic machinery. The components and mechanisms underlying such a signaling system remain largely unknown, but emerging evidence has implicated several receptor-like kinases as regulators of cell wall function.

Plant cell walls are composite structures composed primarily of cellulose and matrix polysaccharides such as hemicelluloses and pectins (Somerville et al., 2004). In addition to wall polymers, structural proteins provide a quantitatively small, but important

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contribution to the wall. The major load-bearing components of the cell wall are the cellulose microfibrils which, in a longitudinally expanding cells, are deposited primarily inan orientation perpendicular to the axis of expansion, thus constricting radial expansion (Green, 1980; Taiz, 1984; Baskin, 2005).

Consistent with a role in differential cell expansion, cellulose-deficient mutants and seedlings treated with inhibitors of cellulose synthesis display reduced or no growth anisotropy, and this is generally accompanied by cell and organ swelling (Somerville, 2006). The oriented deposition of cellulose is guided by underlying cortical microtubules, and thus cortical microtubules are thought to be key determinants of anisotropic growth (Baskin, 2001; Paredez et al., 2006; Lucas and Shaw, 2008).

In the primary cell wall, cellulose is synthesized at the plasma membrane by a hexameric protein complex called cellulose synthase (CESA). Each hexamer is comprised of six CESA proteins that each synthesize a β-1,4-linked glucan chain. A combination of expression analyses, genetic studies, and co-immunoprecipitation experiments have defined roles for the various CESA isoforms in Arabidopsis. CESA1, CESA3, and CESA6 interact with each other to form a class of rosettes that function in primary cell wall biosynthesis (Desprez et al., 2007). CESA2, CESA5 and CESA9 also likely function in primary cell wall synthesis in a manner such that they are partially redundant with CESA6 at different stages of growth (Desprez et al., 2007; Persson et al., 2007). CESA4, CESA7, and CESA8 comprise a distinct subset of rosettes that function in secondary cell wall biosynthesis (Taylor et al., 2000; Taylor NG, 2003). Single mutants in any one of the genes encoding these CESA proteins are deficient in

cellulose biosynthesis, which suggests that a functional cellulose synthase complex requires contributions from three different CESA subunits (Desnos et al., 1996; Arioli et al., 1998; Taylor et al., 1999; Fagard et al., 2000; Taylor et al., 2000; Cano-Delgado et al., 2003; Taylor, 2008).

The same structure and composition that lends strength and rigidity to the wall also serves to constrain cell expansion. While cell wall loosening is essential for expansion, this must be balanced with polymer synthesis and wall re-strengthening to prevent the cell wall from rupturing. Such wall remodeling is facilitated by the activity of loosening and strengthening agents that modify cell wall polysaccharides. For example, wall loosening is accomplished through the activities of hydroxyl radicals, expansins, xyloglucan endoglucosylase/hydrolases, and endog-(1,4)-ß-D-glucanases, whereas the extensins and peroxidases function in wall rigidification (Cosgrove, 2005; Humphrey et al., 2007). Coordinating wall loosening with wall strengthening activities during cell expansion requires the ability of the cell to monitor changes in wall integrity and to signal back to regulate the machinery involved in the synthesis and modification of the cell wall components.

Such a cell wall signaling system has been well characterized in the yeast *Saccharomyces cerevisiae* (Levin, 2005). In this system, the WSC and MID2 cell surface receptors function as sensors of cell wall integrity. Both WSC and MID2 contain an extracellular domain rich in Ser/Thr residues, a single transmembrane domain, and a small carboxy terminal cytoplasmic domain that interacts with the guanine exchange factors, ROM1 and ROM2 (Philip and Levin, 2001). Upon activation, ROM1 and ROM2

stimulate the small GTP binding protein Rho1, which in turn initiates a variety of processes, including changes in the synthesis of β-glucan, nucleation of actin filaments, secretory vesicle targeting, and activation of a MAP kinase cascade that leads to changes in gene expression related to cell wall biogenesis (Ozaki et al., 1996; Levin, 2005).

The hypothesis that plant cells have the ability to sense and respond to changes in wall integrity is supported by the observation that genetic or chemical perturbation of cellulose biosynthesis results in an ectopic deposition of lignin. Lignification, which increases the rigidity of the cell wall, normally occurs in the secondary cell walls of the vascular tissue and in response to pathogen attack. Ectopic lignin deposition has been observed in several cellulose deficient mutants (Vance, 1980), including *rsw1 (root swelling1,) eli1* (*ectopic lignification*)*,* and *prc1 (procuste),* which disrupt *CESA1*, *CESA3* and *CESA6* respectively, as well as the *kor (korrigan)* and *fei* mutants (Nicol et al., 1998; Fagard et al., 2000; Williamson et al., 2001; Cano-Delgado et al., 2003; Xu et al., 2008). Ectopic lignin deposition also occurs in seedlings treated with the cellulose synthesis inhibitors 2,6-dichlorobenzonitrile (DCB) and isoxaben (IXB) (Caño-Delgado et al., 2003). In addition to increased lignin deposition, disruption of cellulose synthesis also results in other changes, including changes in gene expression, activation of ethylene and jasmonic acid signaling pathways, and the inhibition of cell elongation (Caño-Delgado et al., 2003; Duva and Beaudoin, 2009). These changes in cellular function indicate that the cell not only senses changes in the wall, but that there is a feedback system in place to maintain cell wall integrity.

Relatively little is known about the molecular components and signal transduction pathways involved in the regulation of plant cell wall function. Recent studies have implicated multiple receptor like kinases (RLKs) in cell wall signaling. RLKs represent a large (~600 in Arabidopsis), diverse family of proteins (Shiu and Bleecker, 2001) that physically link the cell wall to the cytoplasm, making them ideal candidates for cell wall sensors. RLKs are situated at the plasma membrane and contain an extracellular domain, a transmembrane domain, and an intracellular serine/threonine kinase domain. They have been implicated in various signaling pathways, including meristem function, brassinosteroid perception, floral abscission, ovule development and embryogenesis, plant defense, and overall plant morphology (Becraft, 2002). This review highlights the role of RLKs in cell wall function.

**The Wall Associated Kinases (WAKs)**

The wall-associated kinases (WAKs) are a set of RLKs that are tightly bound to the cell wall (He et al., 1996). There are five highly conserved *WAK* genes in Arabidopsis, and an additional 26 *WAK*-like genes that encode proteins with divergent extracellular domains (Verica et al., 2003). The WAK proteins consist of an extracellular domain, a transmembrane domain, and a cytoplasmic serine/threonine protein kinase domain. The extracellular domains of the WAKs are 40%-60% identical to each other and contain two epidermal growth factor (EGF)-like repeats. This domain binds tightly to pectin in a calcium-dependent fashion (Decreux and Messiaen, 2005). This association with pectin first occurs in an endomembrane compartment, most likely the Golgi (Kohorn et al., 2006). The intracellular kinase domains of the WAKs are more highly

conserved than their extracellular domains, which might reflect similar downstream targets or, alternatively, this catalytic domain may be more evolutionarily constrained. All five WAKs are expressed widely throughout the plant in the expanding cells of leaves, stems, roots, and fruits, and their expression is differentially regulated by environmental and developmental cues such as wounding, pathogen infection, and aluminum (He et al., 1998; Wagner and Kohorn, 2001; Sivaguru et al., 2003).

WAKs are required for cell expansion during plant development. Disruption of WAK function using inducible expression of full length *WAK2* anti-sense RNA, which likely disrupts multiple WAKs, compromised leaf cell expansion (Lally et al., 2001; Wagner and Kohorn, 2001). Consistent with these results, root cell elongation is impaired in *wak2* loss-of-function mutants and in seedlings expressing WAK4 anti-sense RNA (Lally et al., 2001; Kohorn et al., 2006). The growth of a *wak2* loss-of-function mutant was dependent on exogenous sugars, suggesting that the mutation may alter sugar metabolism (Lally et al., 2001; Kohorn et al., 2006). This idea is supported by the finding that *wak2* mutant roots show reduced vacuolar invertase activity, which is critical for the generation of solutes required to maintain turgor pressure during cell expansion (Kohorn et al., 2006). Furthermore, both the expression of *INV1*, which encodes an invertase enzyme, and MAPK3 activity are induced in Arabidopsis mesophyll protoplasts treated with pectin in a WAK2-dependent manner (Kohorn et al., 2009). Loss-of-function *mapk3* mutants, which are aphenotypic, enhanced the phenotypic effects of a WAK2 dominant negative transgene. Together, these results suggest that WAK2 and MAPK3 may be involved in a pathway that modulates the activity of vaculolar invertase by detecting pectin-based signals in the cell

wall (Kohorn et al., 2009).

A combination of *in vitro* and *in vivo* studies have identified a WAK1 protein complex that includes a glycine-rich-extracellular protein (AtGRP-3) and a kinase-associated protein phosphatase (KAPP) (Park et al., 2001). In plants, glycine-rich proteins are considered structural components of the cell wall (Keller, 1993), and thus, in addition to binding pectin, the extracellular domain of WAK1 likely also binds AtGRP-3. The KAPP protein binds to the cytoplasmic kinase domain of multiple receptor kinases in a phosphorylation-dependent manner (Braun et al., 1997; Shah et al., 2002), and in several cases this interaction has been demonstrated to be functionally relevant. AtGRP-3 specifically interacts with WAK1; however, KAPP binds to the kinase domains of both WAK1 and WAK2, as well as to the kinase domains of other RLKs. The expression of *WAK1* and *AtGRP-3* were up-regulated by exogenously added AtGRP-3 protein, suggesting that they are regulated by a positive feedback loop (Park et al., 2001). Although the biological significance of the WAK1/AtGRP-3 interaction has not been determined, the specificity of this interaction, together with the distinct expression patterns of the various *WAK* genes suggests the possibility that the WAKs may sense different signals from the wall.

**The *Catharanthus roseus* RLK1 Like (CrRLK1L) Family**

The *Catharanthus roseus* RLK1 Like family is named after its founding member, CrRLK1, which was identified from the plant *Catharanthus roseus* (Schulze-Muth, 1996). There are 17 members of the Arabidopsis CrRLK1L subfamily of RLKs and four of these have been implicated in regulating cell wall function: FERONIA (FER),

THESEUS1 (THE1), HERCULES1 (HERK1) and HERK2 (Hematy and Hofte, 2008; Guo et al., 2009a; Guo et al., 2009b).

The FER RLK was identified by its role in pollen tube function (Huck et al., 2003). FER-dependent signaling in the synergid cell appears to be required for pollen tube growth arrest and the release of sperm cells in the female gametophyte during fertilization (Huck et al., 2003; Escobar-Restrepo et al., 2007). In *fer* mutant ovules, pollen tubes fail to cease growth and to rupture upon reaching the micropylar entrance of the embryo sac; instead, they continue to grow within the embryo sac, thus failing to fertilize the ovule (Escobar-Restrepo et al., 2007). More recent studies have demonstrated that *fer* mutant seedlings display a pronounced decrease in hypocotyl elongation, petiole length, and overall shoot growth when compared to wild-type seedlings, suggesting that FER also regulates cell elongation in these contexts (Guo et al., 2009a).

The *THE1* RLK was identified as a suppressor of the hypocotyl elongation defect of a loss-of-function mutation in the catalytic subunit cellulose synthase 6 ($cesA6^{prc1}$). *the1* was found to also suppress the hypocotyl growth inhibition of a subset of other mutants altered in cell wall function, including $cesA3^{eli1}$ and $cesA^{rsw1}$, and to suppress the ectopic lignin accumulation observed in these cellulose deficient mutants. However, surprisingly, *the1* did not suppress the defect in cellulose biosynthesis of the $cesA6^{prc1}$ mutant. These results suggest that the inhibition of hypocotyl elongation and the ectopic lignin deposition in the $cesA6^{prc1}$mutant is an active response to cell wall defects that requires signaling through the THE1 receptor. Consistent with this, transcriptional

profiling identified thirty-six genes that were altered by *cesA6*$^{prc1}$ in a *THE1*-dependent manner. The *THE1*-dependent genes included two transcription factors, several proteins involved in protecting the cell against oxidative stress, potential pathogen defense proteins, and multiple genes encoding cell wall proteins (Hematy et al., 2007).

Single loss-of-function mutations in *THE1* in an otherwise wild type background did not result in any detectable change in plant growth and development (Hematy et al., 2007), suggesting that THE1 function is only revealed when the cell wall is perturbed. However, recent studies have shown that *THE1* is genetically redundant with other members of the *CrRLK1L* gene family, as combining *the1* with *herk1* and/or *herk2* mutations, single mutants that are also aphenotypic, resulted in strong effects on cell expansion, including decreased petiole length and shoot growth (Guo et al., 2009a; Guo et al., 2009b) similar to the effects of the *fer* mutation. The overall decreased growth in the double *the1 herk1* mutants was found to be a consequence of reduced cell elongation, implicating these RLKs as important regulators of cell expansion within the cell wall.

Interestingly, six of the 17 *CrRLK1L* genes are regulated by brassinosteroid, including *THE1, HERK1, HERK2 and FER* (Guo et al., 2009a; Guo et al., 2009b). Furthermore, the *herk1 the1* mutations enhanced the dwarfed phenotype of the loss-of-function brassinosteroid receptor mutant, *bri1*, and partially suppressed the excessive cell elongation phenotype of a gain-of-function *bes1*-D mutant (Guo et al., 2009a). Expression profiling of the *the1 herk1* double mutant and the *fer* single mutant suggests these receptors regulate overlapping sets of genes. Furthermore, 16% of the genes

affected in these mutants are regulated by brassinosteroid. These data suggest that while THE1, HERK1, HERK2 and FER may act in a common pathway required for cell elongation, there is crosstalk between this pathway and and the pathway mediating brassinosteroid-regulated cell elongation.

The reduced cell expansion observed in the *the1/herk* multiple mutants at first seems at odds with the increased cell expansion brought about by the *the1* mutation in the *cesA6*$^{prc1}$ background. One simple model to resolve this apparent discrepancy invokes a threshold mechanism: the reduction in THE1/HERK signaling resulting from single *the1* mutations is enough to disrupt the feedback system involved in perception of the altered cell wall function of the *cesA6*$^{prc1}$ mutant, but is not drastic enough to substantially alter basal cell wall synthesis. In contrast, further disruption of this class of receptors (i.e. the *the1 herk* multiple mutants) decreases signaling below a threshold necessary for proper regulation of cell wall synthesis even in basal conditions. The cell must maintain a delicate and dynamic balance between wall rigidity and extensibility during growth, and thus perturbing this proposed feedback system to different levels could shift this balance with distinct outcomes.

**The Leucine-Rich Repeat RLKs (LRR-RLKs)**

The leucine-rich repeat (LRR) RLK family represents the largest group of RLKs encoded by higher plant genomes. The Arabidopsis LRR-RLK family is comprised of 216 genes distributed among 13 different subfamilies (Shiu and Bleecker, 2001). In animals, LRR proteins are important signaling components of many developmental and host defense pathways. However, unlike in plants, animal LRR proteins do not contain a

cytoplasmic protein kinase domain, but instead transduce signals across the plasma membrane by activating co-receptors, a mechanism that may be conserved in plants.

Recently, two LRR-RLKs (FEI1 and FEI2) were demonstrated to play a role in the regulation of cell wall function. Although single *fei1* and *fei2* mutants showed no obvious phenotypes, double *fei1 fei2* mutants displayed conditional root anisotropic growth and ectopic lignin deposition. These phenotypes are characteristic of cellulose deficiency and, indeed, *fei1 fei2* mutant roots displayed a significant decrease in the synthesis of cellulose and possibly other cell wall polymers when grown in non-permissive conditions, suggesting that the FEI receptors regulate the synthesis of cell wall components (Xu et al., 2008). The *sos5* mutant, which was isolated as a mutant that displayed a swollen root tip in the presence of moderately high salt (Shi et al., 2003), was found to have a similar phenotype to *fei1 fei2.* Genetic analysis revealed that SOS5 and the FEIs act through the same pathway to regulate cell wall function (Xu et al., 2008). *SOS5* encodes a cell surface GPI-anchored protein with fasciclin-like domains (Shi et al., 2003), and could act as, or may be involved in, the production or presentation of a FEI ligand.

Both the *fei1 fei2* and *sos5* mutants display swollen root phenotypes only when elevated levels of sucrose or salt are present in the media, which is also observed in several other root swelling mutants, including *cesA6*$^{prc1}$, weak alleles of *cobra* (Xu et al., 2008), and *pom1* and *pom2* mutants (Hauser et al., 1995). This suggests that elevated levels of sucrose or salt sensitize roots to perturbations in cell wall synthesis through an as yet unknown mechanism. It has been suggested that the sucrose-dependent

phenotype of *cobra* and several other root swelling mutants could be linked to the relative rate of root growth, with defects occurring only under conditions of maximal growth rates (Hauser et al., 1995). However, *fei1 fei2* (and *sos5* and weak *cobra* alleles) also display swollen roots on media containing moderately elevated levels of NaCl (Xu et al., 2008), a condition that decreases the rate of root growth.

Further analysis indicated a role for 1-aminocyclopropane-1-carboxylate (ACC) synthase, which catalyzes the rate-limiting step in ethylene biosynthesis, in FEI function. Both FEI1 and FEI2 directly interact with ACS as shown by yeast two-hybrid assays and inhibition of ACC function using either $\alpha$-aminoisobutyric acid (AIB), a structural analog of ACC, or aminooxy-acetic acid (AOA), which inhibits enzymes that require pyroxidal phosphate including ACC synthase, suppressed the root swelling phenotype of both the *fei1 fei2* and the *sos5* mutants. As AOA and AIB block ethylene biosynthesis by distinct mechanisms, it is unlikely that this phenotypic reversion of *fei1 fei2* is due to off-target effects of the inhibitors. Furthermore, this is not a general effect of AIB as it did not revert the root swelling phenotype of the *cobra* mutant (Xu et al., 2008). Surprisingly, inhibition of ethylene perception via mutations or chemical inhibitors had no appreciable effect on the root phenotype of *fei1 fei2* or *sos5-2* mutants (Xu et al., 2008). This suggests that either swelling in the absence of FEI depends on a hitherto undiscovered pathway for ethylene perception, or that ACC itself is acting as a signaling molecule. Consistent with this hypothesis, recent data indicates that, in addition to acting as the immediate precursor to ethylene, ACC itself may also act as an essential regulator of plant growth and development (Tsuchisaka et al., 2009). Genetic disruption of all eight ACC synthase genes in Arabidopsis caused embryonic lethality, in contrast to mutations

that eliminate ethylene perception, such as *etr1* or *ein2*, which have only relatively modest effects on plant development. The precise role of ACC in plant development in general and in the FEI pathway specifically, and how this potential signaling molecule is perceived, are important questions that need to be addressed.

**Conclusions**

While the identification of multiple RLKs that likely play a role in regulating cell wall function is an important begininning in our understanding of cell wall signaling, the field is only in its infancy and many questions remain unanswered. There are over 600 RLKs in Arabidopsis, and it is likely that additional RLKs play a role in regulating wall function. To further enhance our understanding of this signaling system, it is crucial to identify the immediate targets of the RLKs implicated in cell wall function. One potential target could be the cellulose synthase enzyme itself as multiple phosphorylation sites have been identified, clustered primarily in the N-terminal domain, of several CESA proteins (Nuhse et al., 2004; Brown et al., 2005; Persson et al., 2007), and phosphorylation of CESA7 has been linked to its degradation via a 26S proteasome dependant pathway (Taylor, 2007). However, an intact kinase catalytic domain is not required for the function of FEI1/FEI2 (Xu et al., 2008), raising the possibility that, at least for this class of RLKs, the targets may not be regulated solely by phosphorylation. How these RLKs interact with each other and with other signaling pathways to regulate cell wall function is unknown. Finally, while pectin has been identified as a possible ligand for the WAKs, there are no clear candidate ligands for the other RLKs. The near future will likely reveal answers to these and other questions and perhaps an integrated

model describing the mechanisms by which cell walls perceive and respond to signals will emerge.

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# CHAPTER 2

## Alterations in Auxin Homeostasis Suppress Defects in Cell Wall Function

### OVERVIEW

The plant cell wall is a highly dynamic structure that changes in response to both environmental and developmental cues. It plays important roles throughout plant growth and development in determining the orientation and extent of cell expansion, providing structural support, and acting as a barrier to pathogens. Despite the importance of the cell wall, the signaling pathways regulating its function are not well understood. Two partially redundant leucine-rich-repeat receptor-like kinases (LRR-RLKs), FEI1 and FEI2, regulate cell wall function in *Arabidopsis thaliana* roots; disruption of the FEIs results in short, swollen roots as a result of decreased cellulose synthesis. We screened for suppressors of this swollen root phenotype and identified two mutations in the putative mitochondrial pyruvate dehydrogenase E1α homolog, *IAA-Alanine Resistant 4 (IAR4)*. Mutations in *IAR4* were shown previously to disrupt auxin homeostasis and lead to reduced auxin function. We show that mutations in *IAR4* suppress a subset of the *fei1 fei2* phenotypes. Consistent with the hypothesis that the suppression of *fei1 fei2* by *iar4* is the result of reduced auxin function, disruption of both *wei8* and *tar2*, which

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decrease auxin biosynthesis, also suppresses *fei1 fei2*. In addition, *iar4* suppresses the root swelling and accumulation of ectopic lignin phenotypes of other cell wall mutants, including *cesA6*$^{prc}$ and *cobra*. Further, *iar4* mutants display resistance to the cellulose synthesis inhibitor isoxaben. These results establish a role for *IAR4* in the regulation of cell wall function and provide evidence of crosstalk between the cell wall and auxin during cell expansion in the root.

## INTRODUCTION

Cell expansion plays a critical role in plant growth and development. The direction and extent to which cells expand is controlled by the rigid, yet highly dynamic cell wall. The cell wall is a major determinant of cell size and shape and consequently, overall plant morphology. In roots, the architecture of the cell wall permits longitudinal cell elongation while restricting radial expansion, which leads to highly asymmetric, anisotropic growth (Baskin 2005, Green 1980, Steinwand and Kieber 2010, Taiz 1984).

Plant cell walls are composed primarily of load-bearing cellulose microfibrils, cross-linking hemicelluloses, and pectins. Together with a relatively small number of structural proteins, this matrix of polysaccharides lends the wall the strength and rigidity that is required for structural support and plant defense, while simultaneously allowing cells to expand as plants grow and develop (Somerville *et al.* 2004). During cell expansion, wall polymers are actively remodeled and rearranged and their synthesis is altered in response to both developmental and environmental cues (Pilling and Höfte 2003). The ability of cell walls to maintain structural integrity and

function properly as changes in the architecture of the cell wall occur suggests that there is a sensing and feedback system in place to perceive and respond to changes in the wall. Despite a crucial role in the maintenance of plant cell wall function, our current understanding of the components and mechanisms involved in the perception of and response to regulatory input from the wall remains poorly understood.

Several members of the receptor-like kinase (RLK) family have been implicated as sensors of signals from the cell wall. In Arabidopsis, the RLK family is comprised of approximately 600 members, several of which have been implicated in a variety of different signaling pathways that function throughout plant development (Gish and Clark 2011). Of those, members of three different sub-families have been implicated in regulating cell wall function. The wall-associated kinases (WAKs) are tightly bound to the cell wall and are required for normal cell expansion (He *et al.* 1996, Lally *et al.* 2001, Wagner and Kohorn 2001). In addition to the WAKs, four members of the *Catharanthus roseus* RLK1-Like (*Cr*RLK1L) subfamily (*HERCULES1*, *HERCULES2*, *FERONIA*, and *THESEUS1*) and two members of the leucine-rich repeat (LRR) subfamily (*FEI1* and *FEI2*) have been implicated in cell wall signaling. Although members from each of the three RLK subfamilies are required for cell expansion, only *THESEUS1,* it's close homologs, and the FEIs have been linked to cell wall synthesis (Guo *et al.* 2009, Hématy and Höfte 2008, Xu *et al.* 2008).

Mutations in *THESEUS1 (the1)* suppress ectopic lignin deposition and restore hypocotyl elongation in cellulose-deficient mutants, but do not restore cellulose biosynthesis in the *cesA6*$^{prc1}$ mutant (Hématy and Höfte 2008). These data suggest that *THESEUS* plays a role in sensing and actively responding to changes in the cell wall. Disruption of both *FEI1* and *FEI2* leads to a loss of anisotropic growth in rapidly expanding cells of the root elongation zone, but also affects cell expansion in the stamen filament and the hypocotyl of dark-grown seedlings. In addition, the roots of double *fei1 fei2* mutants display ectopic lignin deposition, are hypersensitive to the cellulose synthesis inhibitor isoxaben, and synthesize less cellulose as compared to wild-type roots when seedlings are grow under non-permissive conditions of elevated salt or sucrose (Xu *et al.* 2008). Further, disruption of FEI2 leads to a reduction in the rays of cellulose observed in the mucilage of wild-type seeds. These data suggest that FEI1 and FEI2 positively regulate cell wall function by promoting cellulose synthesis.

The fasciclin-like GPI-anchored extracellular protein SOS5 acts in the FEI pathway to regulate cell wall synthesis (Xu *et al.* 2008). Like *fei1 fei2, sos5* mutants display short, swollen roots when grown under the restrictive conditions of elevated salt or sucrose, and this phenotype is reversed in both mutants by blocking ethylene biosynthesis, but not ethylene perception. Further, SOS5 also regulates the synthesis of cellulose during the production of seed coat mucilage (Harpaz-Saad *et al.* 2011). Introduction of *sos5* into the *fei1 fei2* mutant does not cause an additive phenotype, in contrast to other mutants affecting cellulose biosynthesis such as *cobra*. The non-additive phenotype of *fei1 fei2* and *sos5* mutations suggests that the

FEI RLKs act in a linear pathway with SOS5 to regulate cellulose synthesis (Xu *et al.* 2008). Taken together with studies in the root, these data suggest an important role for the FEI RLKs/SOS5 pathway in positively regulating cellulose synthesis.

In order to better understand the FEI signaling pathway, we sought to uncover additional components involved in regulating cell wall synthesis in the root. Here we describe the identification and characterization of a suppressor of the *fei1 fei2* mutant. We show that mutations in the previously characterized *IAA-Alanine Resistant 4* (*IAR4)* gene, encoding a putative mitochondrial E1$\alpha$ pyruvate dehydrogenase subunit, suppress the defects in root anisotropic cell expansion exhibited by *fei1 fei2*. *IAR4* was originally identified in a forward genetic screen for IAA conjugate-resistant mutants (LeClere *et al.* 2004). *IAR4* was subsequently identified as an enhancer of *tir1* auxin resistance (Quint *et al.* 2009). Although the precise role of *IAR4* in the auxin biosynthesis pathway remains unclear, *iar4* mutants display phenotypes consistent with reduced endogenous auxin, accumulate IAA-amino acid conjugates, and are rescued by increasing endogenous IAA levels in the plant (LeClere *et al.* 2004, Quint *et al.* 2009). Thus, IAR4 is predicted to play an important role in maintaining auxin homeostasis. Here we show that reduced auxin function, via either *iar4* single or a *wei8/tar2* double mutant, suppresses growth isotropy of cell wall mutants, including *fei1 fei2*. Our results shed light on the role of auxin in regulating cell wall function in the Arabidopsis root.

## RESULTS

### Isolation and characterization of *shou2*

In order to identify additional elements regulating cell wall function we screened for suppressors of the swollen root phenotype of *fei1 fei2* mutants. An $M_2$ population of ethyl methanesulfonate mutagenized *fei1 fei2* was screened for suppressors of the conditional short, swollen root phenotype of *fei1 fei2* seedlings. Eight independent suppressor lines that retested as robust *fei2 fei2* suppressors were identified from screening approximately 200,000 $M_2$ seedlings representing 30,000 $M_1$ seeds. We designated these suppressors *shou* mutations (the Chinese word for thin). These suppressors represented seven distinct loci, two of which were allelic and were designated *shou2-1 and shou2-2*. The *fei1 fei2 shou2*-1 and *fei1 fei2 shou2*-2 lines both had significantly fewer and shorter root hairs. The $F_1$ of a backcross to the parental *fei1 fei2* line displayed a non-suppressed phenotype, and the suppressor phenotype segregated in a ratio of 3 non-suppressed: 1 suppressed in the $F_2$ progeny of this backcross, consistent with *shou2* acting as a single locus, recessive mutation. In addition to the suppression of root length (Fig. 1B), the *shou2* mutations also suppress the radial swelling (Fig. 1A) and the radial expansion of cells in the elongation zone (Fig. 1C) in *fei1 fei2* roots. We isolated the *shou2-1* mutation by backcrossing to the wild type. This *shou2-1* single mutant line displayed fewer and shorter root hairs, similar to the *fei1 fei2 shou2*-1. Intriguingly, under the non-permissive conditions used to assess the *fei* phenotype (grown on MS + 4.5%

sucrose), both the *fei1 fei2* and *shou2-1* parental seedlings displayed roots that were significantly shorter that their wild-type counterparts, despite the fact that the *fei1 fei2 shou2-1* triple mutants displayed nearly wild-type root elongation in the growth condition that was used for the suppressor screen.

### ***SHOU2* is allelic to *IAR4***

We used a map-based positional cloning approach to isolate the *SHOU2* gene. The *fei1* and *fei2* mutations (isolated in the Columbia (Col) ecotype) were introgressed six times into the *Landsberg erecta* (Ler) ecotype to generate a *fei1 fei2* plant that was largely Ler except for small regions of DNA near the *fei1* and *fei2* mutations (see Methods). This line was crossed to *fei1 fei2 shou2* to generate a mapping population for *shou2-1*. Mapping with Col/Ler SSLPs indicated that *SHOU2* was linked to the top of chromosome 1. Analysis of 350 *fei1 fei2* $F_2$ progeny with additional molecular markers further delimited *SHOU2* to a 47 kb interval between the F3I6.D and F3I6.F markers (Fig. 2A; Table 2.1). Sequencing of candidate genes within this region identified missense mutations in the first and seventh exon of *IAR4* (AT1G24180) of *fei1 fei2 shou2-1* and *fei1 fei2 shou2-2* respectively. The *shou2-1* allele contains a C→T transition in the fifth exon of the coding region of *IAR4*, which converts an arginine residue to a stop codon. The *shou2-2* mutation is the result of a G→A transition that is predicted to change a glutamate at position 366 to a stop codon (Fig. 2B). To confirm that *shou2* mutations correspond to AT1G24180, we examined the ability of an independent T-DNA insertional allele that contains a T-DNA insertion in the first exon of *IAR4* (SALK_091909) to suppress *fei1 fei2.* This *shou2*-3 allele was introduced into a *fei1 fei2* mutant line by crossing and the

phenotype of the roots was examined in non-permissive conditions (Fig. 2B). Similar to the other alleles, *shou2-3* suppressed the root swelling phenotype of *fei1 fei2*, which confirms that mutations in *IAR4* correspond to *shou2*. To avoid confusion and be consistent with the prior studies, we re-named *shou2-1, shou2-2,* and *shou2-3,* to *iar4-5, iar4-6,* and *iar4-7* respectively.

**A role for auxin in regulating cell wall function**

As *IAR4* is involved in the maintenance of auxin homeostasis and mutations in *IAR4* restore anisotropic growth in *fei1 fei2*, we hypothesized that a reduction in the level of endogenous IAA would also suppress the loss of growth anisotropy in *fei1 fei2*. To test this hypothesis, we examined the effect of mutations in the auxin biosynthetic genes *WEI8* and *TAR2* on the *fei1 fei2* root swelling phenotype. *WEI8* and *TAR2* are partially redundant genes that encode two of the five tryptophan aminotransferases (TAA1) essential for the major auxin biosynthesis pathway in plants. The level of IAA in the roots of double *wei8 tar2* mutants is reduced by 50% relative to the wild type (Stepanova *et al.* 2008), suggesting *WEI8* and *TAR2* are required for auxin biosynthesis in roots. We generated a *wei8 tar2 fei1 fei2* quadruple mutant to examine whether significant reductions in endogenous auxin levels in the root suppressed growth isotropy in *fei1 fei2*. When grown under restrictive conditions, the swelling of the root tip was suppressed in the quadruple *wei8 tar2 fei1 fei2* mutant (Fig. 3). The suppression of the *fei1 fei2* phenotype by *wei8* and *tar2* is similar to the suppression of *fei1 fei2* by *iar4* and suggests that auxin is required for the radial cell expansion that occurs in response to decreases in cellulose synthesis in the absence of the FEI proteins.

To further explore this hypothesis, we investigated the sensitivity of the *iar4-5* mutant to the cellulose synthesis inhibitor isoxaben. Previous work has shown that loss of growth anisotropy is exacerbated in cell wall mutants treated with isoxaben (Desprez *et al.* 2002, Scheible *et al.* 2001). Consistent with these results, *fei1 fei2* is hypersensitive to isoxaben (Xu *et al.* 2008). However, in contrast to *fei1 fei2*, both the triple *fei1 fei2 iar4-5* and single *iar4-5* are partially resistant to the effects of isoxaben on root swelling (Fig. 4). The suppression of aberrant cell expansion by *iar4-5* suggests that the effect of the loss of cell wall integrity on root morphogenesis can be attenuated by a reduction in auxin function.

**The effect of *iar4* on other *fei1 fei2* phenotypes**

We have previously shown that the FEI RLKs are required for proper hypocotyl cell expansion in etiolated seedlings and in anchoring pectin in seed coat mucilage to the seed surface (Harpaz-Saad *et al.* 2011). The hypocotyls of dark-grown *fei1 fei2* seedlings are significantly wider than those of the wild type (Xu *et al.* 2008). In addition, mutations in *FEI2* lead to disruption of seed coat mucilage structure (Harpaz-Saad *et al.* 2011). We examined whether mutations in *IAR4* could suppress these additional *fei1 fei2* phenotypes. In contrast to its role in the root, *iar4* did not suppress the increased hypocotyl width phenotype of *fei1 fei2* (Fig. 5A and 5B). In fact, the *iar4-5* mutant also had slightly wider hypocotyls and this effect was additive with that of *fei1 fei2*. The additive nature of *iar4-5* and *fei1 fei2* on hypocotyl width suggests that these genes may act in parallel to regulate cell wall function. Unlike in the hypocotyl, mutations in *iar4-5* did not affect the seed coat mucilage of *fei1 fei2.* The seed coat mucilage of the triple *fei1 fei2 iar4-5* mutant resembled that

of *fei1 fei2* indicating that mutations in *IAR4* do not suppress this phenotype (Fig. 6A and 6B).

An additional role for the FEI RLKs is to act additively with COBRA (COB) in stamen filament elongation in the flower. *COBRA* encodes a GPI anchored protein that associates with the cell wall and is required for the oriented deposition of cellulose in rapidly expanding cells (Roudier *et al.* 2005). Like *fei1 fei2, cob-1* mutants are deficient in cellulose and as a result display a short, swollen root phenotype that is enhanced by elevated sucrose. Although neither the *fei1 fei2* nor *cob-1* mutants themselves display an obvious floral phenotype, a triple *fei1 fei2 cob-1* mutant has short stamen filaments and as a result is partially infertile (Xu *et al.* 2008). Similar to root cells in the elongation zone, cells of the stamen filament also undergo primarily longitudinal expansion. Therefore, we assessed the ability of *iar4-5* to suppress the short stamen phenotype of *fei1 fei2 cob-1* mutant. Analysis of a quadruple *fei1 fei2 cob-1 iar4-5* mutant indicated that the *iar4-5* allele restores stamen filament length and fertility in *fei1 fei2 cob-1* (Fig. 5C). Thus, *iar4-5* suppresses some, but not all of the *fei1 fei2* phenotypes.

### ***iar4*** **is a general suppressor of defects in cell wall synthesis**

To ascertain whether loss-of-function mutations in *IAR4* suppress defects in cell expansion exhibited by other cell wall mutants or whether they are specific to the FEI pathway, we crossed *iar4-5* to *sos5, procuste (prc;* a hypomorphic allele of *cesa6*), and a weak allele of *cobra*, *cob-1.* When grown in the presence of 4.5% sucrose, each of these mutants displayed a substantial reduction in root length

accompanied by radial expansion of cells in the root tip as a result of reduced cellulose biosynthesis. As expected, *iar4* suppressed the defects in cell expansion that occur in the *sos5* mutation, which acts in the FEI pathway. However, in contrast to the f*ei1 fei2 iar4* triple mutant that displayed a substantial suppression of the root elongation defect observed in both parental lines, the *sos5 iar4* double mutant retains a reduced root elongation phenotype. Thus, the root of the *iar4 sos5* double mutant is short, but not swollen and thus resembles the *iar4* parental root phenotype. *iar4-5* also suppresses the swollen root phenotypes of both the *cob-1* and *prc* mutants, both of which affect cellulose synthesis independent of the FEI pathway (Fig. 7).

We next tested whether mutations in *IAR4* could suppress the accumulation of ectopic lignin in these mutants. Lignin is deposited ectopically into the cell wall in response to decreased cellulose synthesis that occurs in cellulose deficient mutants. Previous studies have shown that the roots of *fei1 fei2, cob-1,* and *prc* all accumulate ectopic lignin (Caño-Delgado *et al.* 2003, Desprez, *et al.* 2002, Fagard *et al.* 2000, Xu *et al.* 2008). Interestingly, when we assessed the roots of these cell wall mutants in an *iar4-5* background using a colorimetric stain, no ectopic lignin deposition was observed (Fig. 8). This result is consistent with the suppression of the root swelling defect in these mutants by *iar4* and suggests that IAR4 is required for the ectopic deposition of lignin that occurs in response to decreased cellulose biosynthesis. Taken together, these observations suggest that *iar4* is not specific to the FEI pathway, but rather acts as a more general suppressor of defects in cellulose biosynthesis.

## DISCUSSION

We demonstrate that reducing auxin function, either through loss-of-function mutations in *IAR4* or in the auxin biosynthetic genes *WEI8* and *TAR2,* suppresses the root swelling that occurs in the *fei1 fei2* mutant. Several lines of evidence suggest that *iar4*, and by inference, auxin, acts not in the FEI pathway directly, but rather independently to regulate cell wall function. First, *iar4* acts additively with *fei1 fei2* to increase hypocotyl width. Second, *iar4* reverts the swollen root phenotype and suppresses the accumulation of lignin ectopically in other cellulose synthesis mutants such as *cob-1* and *prc,* which act in parallel with the FEIs. Finally, the *iar4* mutation confers resistance to the cellulose synthesis inhibitor, isoxaben. The data support a model in which reduced auxin function acts to modulate cell wall function in the root in some way to counteract the effects of reduced cellulose synthesis.

Previous studies have linked auxin to the regulation of cell wall function. The acid-growth hypothesis attributes auxin-induced cell expansion to the acidification of the cell wall and resulting increase in activity of the wall loosening enzymes expansins (Hager 2003). Expansins disrupt the non-covalent bonds that form between cellulose and hemicelluloses in the wall and thus promote cell expansion in hypocotyls and modulate the growth of leaves, petioles, and roots (Cosgrove *et al.* 2000, Cosgrove *et al.* 2002, Hager 2003) Auxin Binding Protein (ABP1) may play an important role in this response; ABP1 activates $H^+$ ATPases and $K^+$ channels at the plasma membrane upon the perception of auxin and is required for cell elongation (Sauer and Kleine-Vehn 2011). In addition to expansins, other wall-loosening

enzymes such as xyloglucan hydrolases (XGH) and endotransglycosylases (XET), which cleave and re-graft a major form of hemicellulose, xyloglucan, are also activated upon acidification of the cell wall and in response to auxin (Lorences and Zarra 1987). Consistent with these findings, the mechanical extensibility of epidermal cells isolated from azuki bean epicotyls increases dramatically following incubation with XGH (Kaku *et al.* 2002). These are among many studies that support a role for auxin in increasing the extensibility of the cell wall in the shoot.

The role of auxin in roots is less well studied. The notably shorter root of the auxin-insensitive, gain-of-function Aux/IAA mutant, *axr3-1,* coupled with the repression of numerous genes involved in cell wall synthesis and remodeling suggests a similar role for auxin in regulating cell wall function in the root to that of the shoot. Among the genes that are de-regulated in *axr3-1* seedlings treated with IAA are those that encode arabinogalactan proteins (AGPs), expansins (EXP), extensins, proline rich proteins (PRP), xyloglucan endotransglucosylase-hydrolases (XTHs), and pectin methyl-esterases (PMEs) (Overvoorde *et al.* 2005). Although extensins rigidify the cell wall, a disproportionate number of genes repressed in *axr3-1* encoded proteins that loosen the cell wall matrix and thus promote cell elongation (Cosgrove 2005). Similarly, mutations in the auxin influx carrier, *lax3*, prevent the induction of expansin expression in the root of Arabidopsis seedlings in developing lateral roots. *LAX3* is required for lateral root initiation and its expression precedes the necessary changes in cell wall architecture that are predicted to play a critical role in the emergence of lateral root primordium (Swarup *et al.* 2008). The lack of wall plasticity coupled with alterations in the expression of genes that encode

cell wall remodeling proteins in the *axr3-1* and *lax3* mutants provide further evidence that auxin promotes wall loosening.

The growth of seedlings in the presence of auxin has been shown to lead to root swelling in a manner independent of ethylene biosynthesis (Alarcon *et al.* 2012, Eliasson *et al.* 1989). This suggests that exogenous auxin decreases the integrity of the cell wall, leading to a loss of growth anisotropy. This is consistent with the suppression of swelling in mutants defective in cellulose biosynthesis by reduction of endogenous auxin that is described here. An important question is by what the mechanism does exogenous auxin increases root swelling in wild-type roots, and conversely, how does reduced endogenous auxin suppress swelling in cellulose-deficient roots. One possibility is that auxin negatively regulates cellulose synthesis. The suppression of the *procuste* mutant by *iar4* makes this somewhat unlikely as *procuste* is a null allele of CESA6. However, it is possible that reduced auxin levels may elevate cellulose synthesis via alternative CESA complexes as CESA6 acts redundantly with CESA2, CESA5, and CESA9 in some Arabidopsis tissues. This scenario is unlikely, at least with respect to CESA5 because mutations in *IAR4* do not suppress the defects in seed coat mucilage production in *fei1 fei2* where CESA5 is required for cellulose biosynthesis. A somewhat more plausible model is that auxin modulates the rigidity of the wall not by regulating cellulose synthesis, but by altering other properties of the wall, such as the crosslinking of cellulose microfibrils or the activity of extensins or other cell wall modifying enzymes as described above. Intriguingly, while both *iar4* and *fei1 fei2* mutant roots are short in the presence of elevated sucrose, the triple *iar4 fei1 fei2* mutant displays both a non-swollen root, as

well as root elongation comparable to the wild type. In contrast, although mutations in *iar4* suppress the swollen root phenotypes of the other cell wall mutants, they do not restore root elongation in *prc, cob,* or *sos5.* This suggests that *iar4* does not simply restore cellulose biosynthesis in these mutants, as this would rescue both the swollen root and root length phenotypes. Additionally, it is conceivable the short root phenotype of *iar4* may be partially attributed to decreased cell division in the root apical meristem as auxin has been shown to regulate both the activity and the size of the meristem in the Arabidopsis root. However, although we isolated *iar4* as a suppressor of *fei1 fei2,* the short root phenotype of both *fei1 fei2* and the *iar4* mutant but not the *fei1 fei2 iar4* triple mutant raises an interesting question. How does *fei1 fei2* suppress *iar4*? It is unlikely that it does so by suppressing any potential effects of iar4 on cell division rates, and thus likely does so through modulation of cell wall properties.

We propose a model that combines the previously characterized role of the FEI receptor-like kinases in regulating cellulose synthesis with a role for auxin in regulating cell wall rigidification in the root (Fig 9). We have previously shown that ACC may act as a signal in the FEI pathway to regulate cellulose biosynthesis. The *fei1 fei2* mutations lead to radial cell expansion in the root as a result of decreased cellulose synthesis, which alters wall function such that there is not sufficient force to constrict radial expansion. One model consistent with the data is that decreased auxin results in an increase in the rigidity of the cell wall, which can restrict radial cell expansion in cellulose-deficient mutants. In most cases this increase in rigidity would also cause a decrease in the overall expansion of the cells, and hence a decrease in

the length of the root. In the case of the *fei1 fei2 iar4* line, this decreased cellulose coupled with the increased rigidity caused by decreased auxin is precisely balanced, leading to both a lack of swelling and near wild-type elongation. Auxin also increases the expression of multiple ACS genes (Abel *et al.* 1995), which may also have an effect on the FEI signaling pathway.

Alternatively, auxin could be involved in the signaling cascade linking perception of perturbation of the cell wall to changes in cell wall synthesis. Interestingly, a recent study has demonstrated that the inhibition of root cell elongation that occurs in response to isoxaben is attenuated by mutations in the *tir1-1* auxin receptor and growth in the presence of the synthetic antagonist of TIR1, PEO-IAA. Furthermore, results from this study indicated that inhibitors of the precursor to ethylene, ACC, fully restore growth anisotropy in the presence of isoxaben and this effect was shown to act independent of ethylene (Tsang *et al.* 2011). Consistent with this data, inhibitors of ACC, but not ethylene suppress the swollen root phenotype of *fei1 fei2* (Xu *et al.* 2008).

The characterization of *iar4* in this study as a suppressor of defects in cell wall synthesis provides evidence that auxin plays a key role in the regulation of primary cell wall function and suggests that wall extensibility may be a major determinant of cell expansion in the root. Whether auxin acts as a general regulator of cell wall function throughout development or participates in the active signaling processes that occur in response to perturbations in the cell wall remains an interesting question for future studies.

## CONTRIBUTIONS

We thank Jose Alonso at North Carolina State University for *wei8 tar2-1* seeds and Yujin Sun for help with the photography. This project was supported by grants IOS 0624377 and MCB-1021704 from the National Science Foundation to JJK.

## MATERIAL AND METHODS

### Plant Material and Growth Conditions

All lines used in this study are in the Columbia (Col-O) ecotype of *Arabidopsis thaliana,* except where noted. The *shou2-3* (SALK_091909) allele was obtained from the SALK T-DNA insertional collection (Alonso *et al.* 2003). The *prc-1* (Fagard *et al.* 2000) and *cob-1* (Schindelman *et al.* 2001) mutants were obtained from the Arabidopsis Stock Center. The *wei8* and *tar2-1* mutants have been previously described (Stepanova *et al*. 2008). For in vitro studies, seeds were surface sterilized, cold treated for 4 days at 4°C, germinated on vertical plates containing 1 x Murashige and Skoog (MS) salts , 0.6% phytagel (Sigma, St Louis, MO, USA) and either 0% or 4.5% sucrose and grown at 22°C under constant light. For the analysis of root elongation, seeds were germinated on 4.5% sucrose and total root elongation was measured after 14 days. For the hypocotyl elongation assay, seedlings were exposed to light for 3 hours and grown for 4 days in the dark on MS agar supplemented with 1% sucrose. The width of each hypocotyl was measured 1 mm from the hook of an etiolated seedling. For growth on soil, plants were grown either under constant light or long day conditions at 23°C. For growth in the presence of

isoxaben, seedlings were germinated and grown in the absence of sucrose for 5 days then transferred to MS agar supplemented with 0 nM or 5 nM isoxaben for 24 hours.

**Positional Cloning of *shou2***

The *fei1* and *fei2* mutations (Columbia, Col ecotype) were introgressed into Landsberg erecta (Ler) through back crossing with Ler six times and a line homozygous for *fei1* and *fei2* was obtained. Theoretically, after six backcrosses, approximately 98.4% of the genome is Ler, with the exception of regions around the *fei1* and *fei2* mutations, which remain Col. We tested 42 molecular markers across all 5 chromosomes and found only the molecular markers F6NI8 and TI0P12 (close to FEI1), and TIJ8 (close to FEI2) remained Col. All other 39 markers were homozygous for the Ler SNPs. A mapping population was generated by crossing *fei1 fei2 shou2-1* (Col) to *fei1 fei2* (Ler). Bulk segregant analysis was performed using a total of 42 markers that span the Arabidopsis genome on a pool of DNA obtained from 40 $F_2$ seedlings showing suppression of the *fei1fei2* phenotype. The mutation was initially mapped to an interval spanning markers FI2K8 (7.954Mbp) and FI3K9 (9.744Mbp) on chromosome 1. Fine mapping was facilitated by the root hair phenotype of *shou2* mutants using restriction fragment length polymorphisms and cleaved amplified polymorphic sequence markers. The *shou2-1* mutation was mapped to a ~47-kilobase (kb) region delimited by recombination events between marker F3I6-D (8.552 Mbp) and F3I6-F (8.599Mbp) of chromosome 1. Sequencing of 12 genes within this region identified mutations in the first and seventh exons of At1g24180 in *fei1 fei2 shou2-1* and *fei1 fei2 shou2-2* respectively.

**Phloroglucinol Staining**

Seedlings were fixed in a solution of three parts ethanol: one part acetic acid for fifteen minutes and transferred to 70% ethanol for 10 minutes. Seedlings were then cleared in chlorohydrate:glycerol:water (8:1:2) for 5 minutes and stained for a total of 5 minutes in a 2% phloroglucinol-HCl solution.

**Microscopy and seed staining**

The calcofluor stain was done as described by Willats *et al.* (2001). Seeds were pre-treated with 50mM EDTA, stained for 20 min in 25 μg/ml fluorescent brightener 28 (Sigma), washed overnight in water and then visualized using a Zeiss LSM710 confocal microscope equipped with a 405 nm laser diode. Pontamine staining was done as described by (Anderson *et al.* 2010).Seeds were stained for 30 min in 0.01% pontamine fast scarlet S4B (Sigma) following a 90 min pre-hydration, washed for 4 hours in water and then visualized using a Zeiss LSM710 confocal microscope equipped with a 561 laser. Flowers and root tips were imaged using bright field microscopy and hypocotyls using dark field microscopy on the compound Leica microscope. Cross sections of the root elongation zone were done as described by Xu *et al*. (2008).

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## TABLES

| Marker | Chr. | Position | Enzyme | Col | Ler | Oligonucleotides (5' to 3') |
|---|---|---|---|---|---|---|
| | | | | Size of predicted product (bp) | | |
| | | | | | | CCAGTTGTTCAGGAAATGGAA |
| F5O8 | 1 | 8.392 | | 145+251 | 396 | TGACGAATGTATTGCAACCG |
| | | | | | | GTGATCTTGCGCCAGAAGTA |
| T23E23D | 1 | 8.442 | *Bsp1286I* | 537 | 346+191 | CAACCTGATTGTCTGCCTCA |
| | | | | | | CCGAACCAACCTTGAATTTG |
| F3I6-E | 1 | 8.530 | | 320 | 134+186 | TTGGTGTGCCGATAAAAACA |
| | | | | | | TGCCATGTCGTAAATTCCTG |
| F3I6-D | 1 | 8.552 | *MseI* | 215+35+23 | 250+22 | GCAGAATAAGCCATCGTGGT |
| | | | | | | TTCAGTTCACGATTAAAATTGCAAT |
| F3I6H | 1 | 8.570 | *BsrDI* | 173 | -20 | TCTTCTCAGCTGTTTCGTCG |
| | | | | | | GGGACCTCGTTACCCAAAAT |
| F3I6F | 1 | 8.599 | *BsrDI* | 524 | 195+329 | GCTTCAACACTCCTCCAAATC |
| | | | | | | TTGTCGAAGGGACAGTGTTG |
| F3I6-C | 1 | 8.607 | | 197 | -15 | GTGGTCTGCTCTCAGCCTCT |

**Table 2.1 Markers used to map *shou2-1.***

## FIGURES

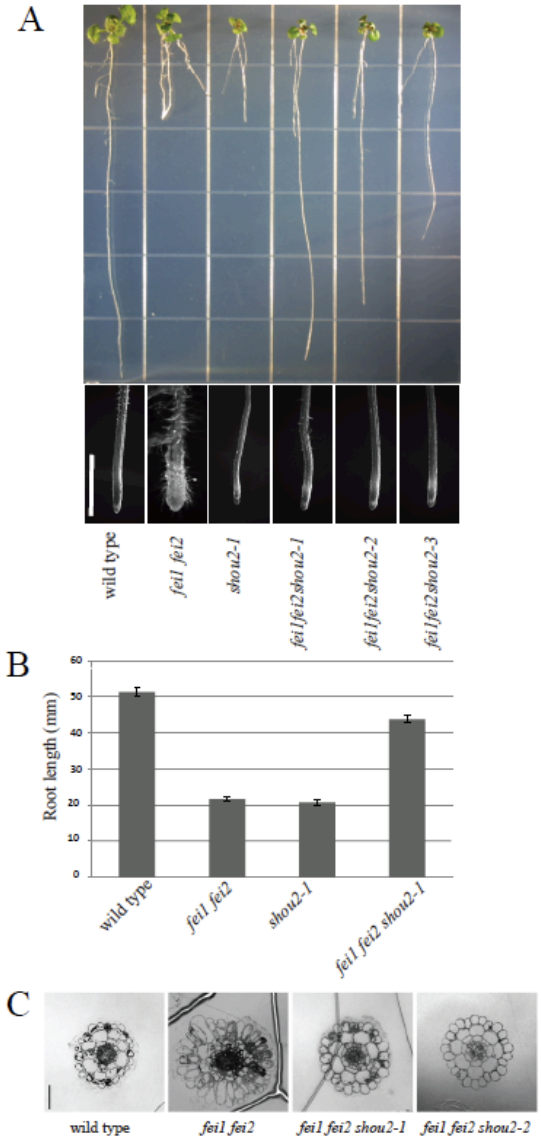


**Figure 2.1. Isolation of the *shou2* suppressor.**

**(A)** Phenotypes of indicated seedlings grown on MS medium containing 4.5% sucrose for three weeks. The bottom panels show a close-up of the root tips. Scale Bar = 1mm.

**(B)** Quantification of total root length from **(A)**. The mean (n-150) ± SE.

**(C)** Transverse sections through the root elongation zone of wild type, *fei1 fei2, fei1 fei2 shou2-1,* and *fei1 fei2 shou2-2.*

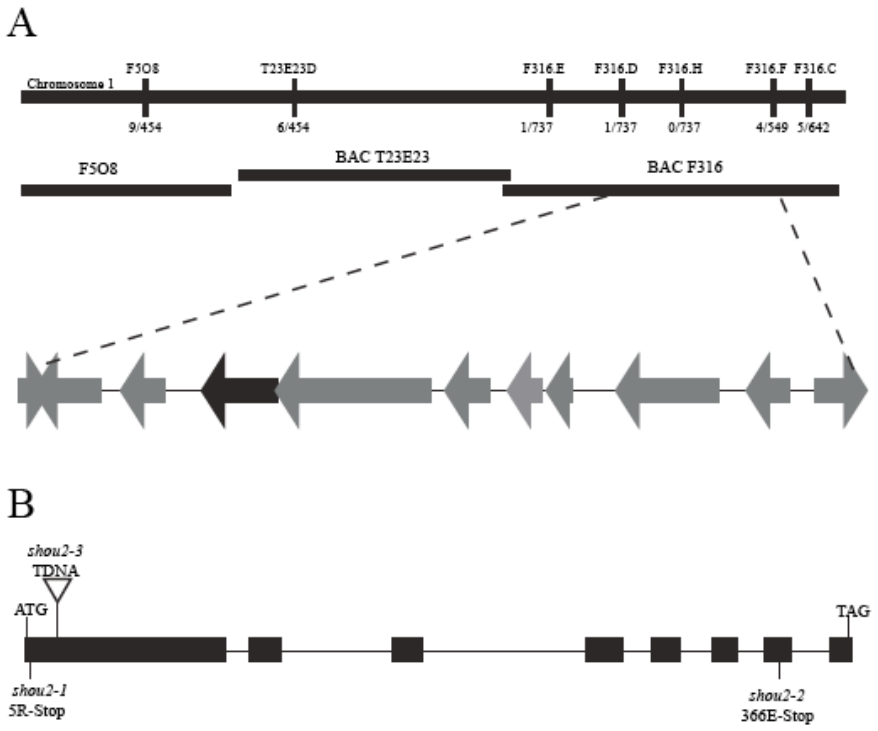


**Figure 2.2 Positional cloning of *shou2.***

**(A)** *SHOU2* was mapped to a region on chromosome 1 between markers F3I6.D and F316.F. The name of each DNA marker is shown above and the number of recombinants is indicated below the line. Open reading frames located between markers F3I6.D and F3I6.F are shown below BACs.

**(B)** Structure of *SHOU2*. Boxes represent exons and the predicted start and stop codons are indicated.. The positions and changes of the three *shou* alleles are indicated.

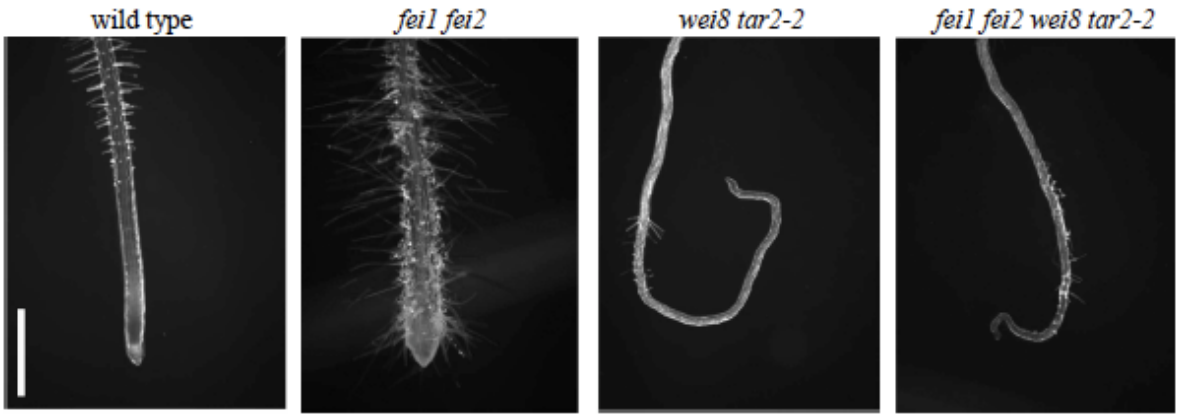


**Figure 2.3 Mutations in the auxin biosynthetic genes *WEI8* and *TAR2* suppress *fei1 fei2.***

Phenotypes of seedlings after transfer at 4 d from MS medium containing 0% sucrose to medium containing 4.5% sucrose. Bar = 1 mm.

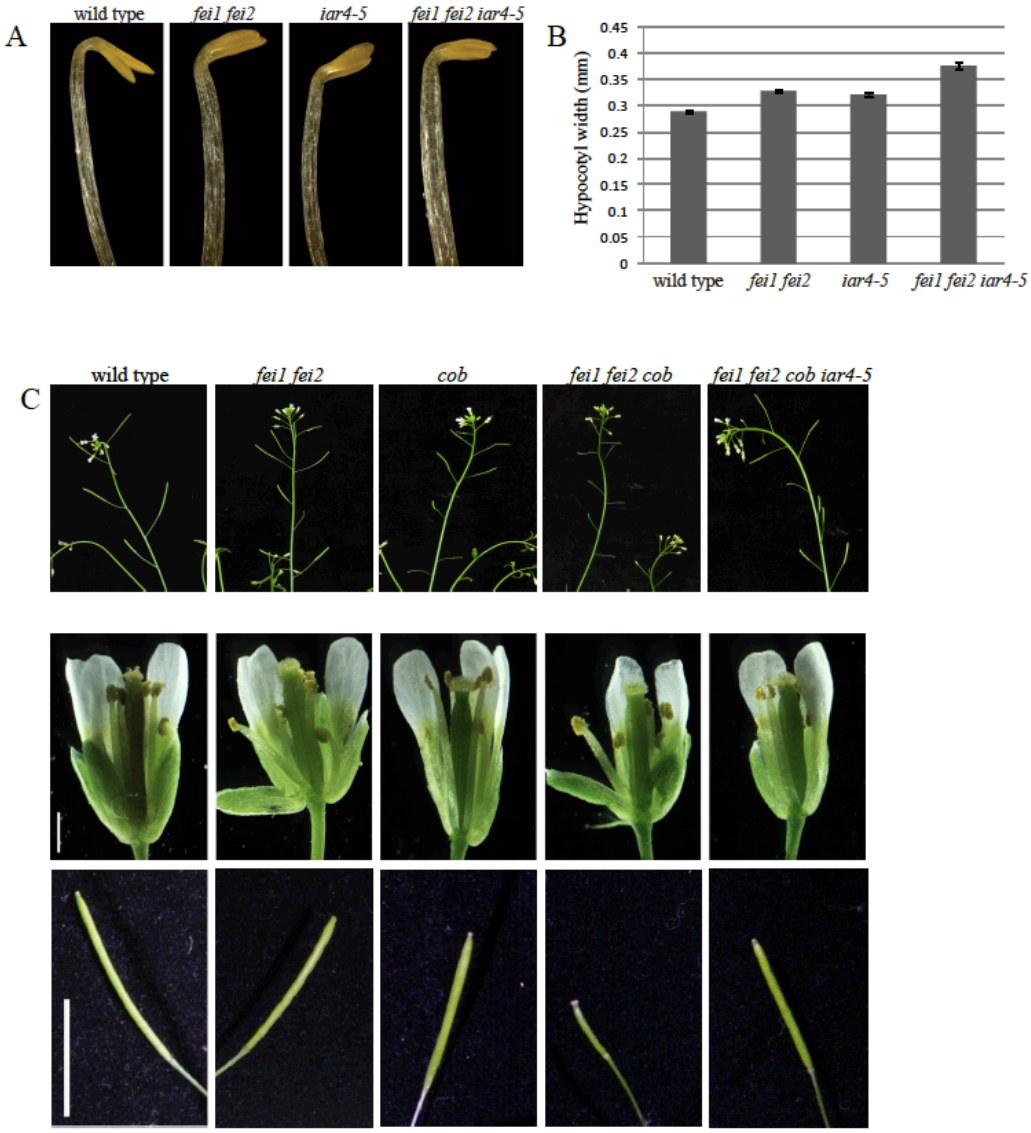


**Figure 2.4 Additional phenotypes of *fei1 fei2 iar4-5*.**

**(A)** and **(B)** Hypocotyls of four-day-old etiolated seedlings of the indictaed genotypes. (A) is an image of a representative seedling and (B) shows the average width (n=20) ± SE.

**(C)** Floral phenotypes from the indicated genotypes. Bar = 1 cm. Plants were grown on soil under long day conditions for four weeks. To visualize stamen length, the petals and sepals were removed from each flower. Bar = 1 mm. Note that the *fei1 fei2 cob* triple mutant is sterile, presumably due to the shortened stamen filaments.

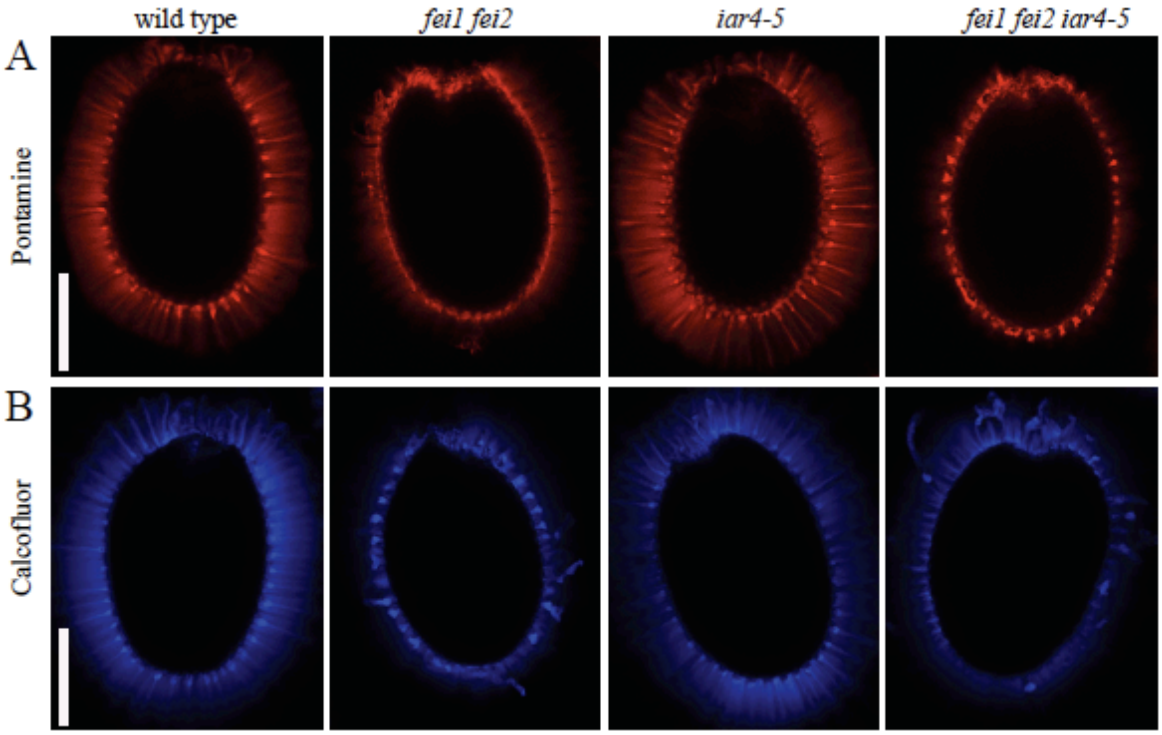


**Figure 2.5 *iar4* does not suppress defects in the seed coat mucilage of *fei1 fei2.***

**(A)** Pontamine stain for cellulose. Bar = .2 mm.

**(B)** Calcofluor stain for cellulose and other cell wall polymer. Bar = .2 mm.

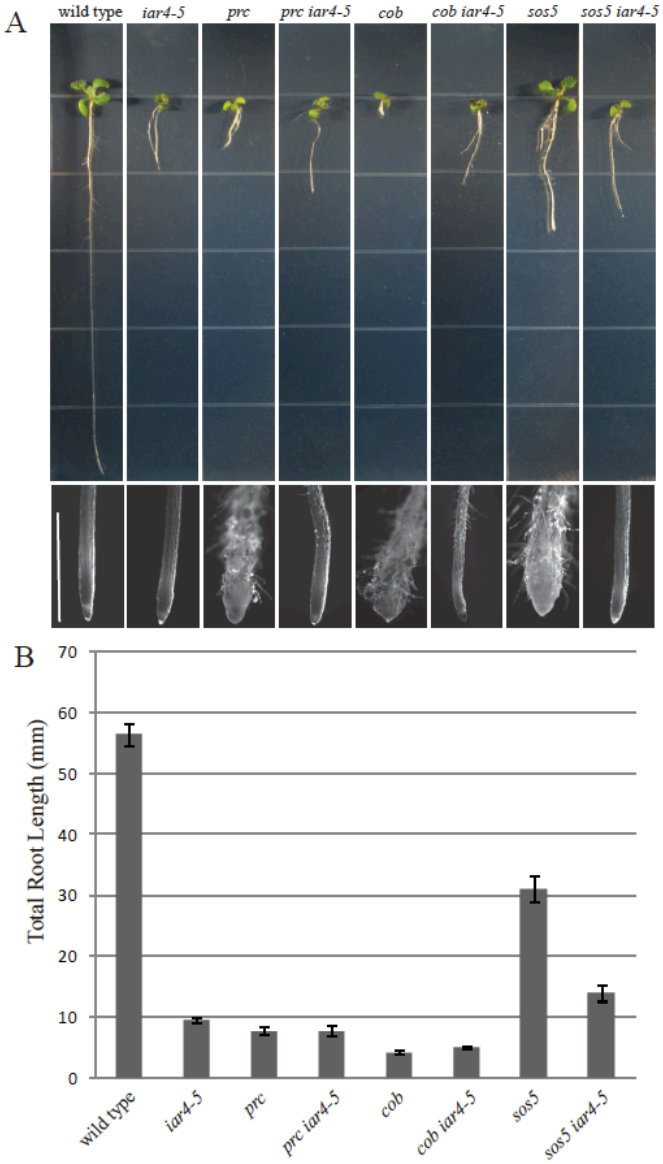


**Figure 2.6 Mutations in *iar4-5* suppress other cell wall mutants.**

**(A)** Phenotypes of indicated seedlings grown on MS medium supplemented with 4.5% sucrose for 20 days. Scale bar = 1.5mm.

**(B)** Quantification of root elongation of seedlings in **(A)**.

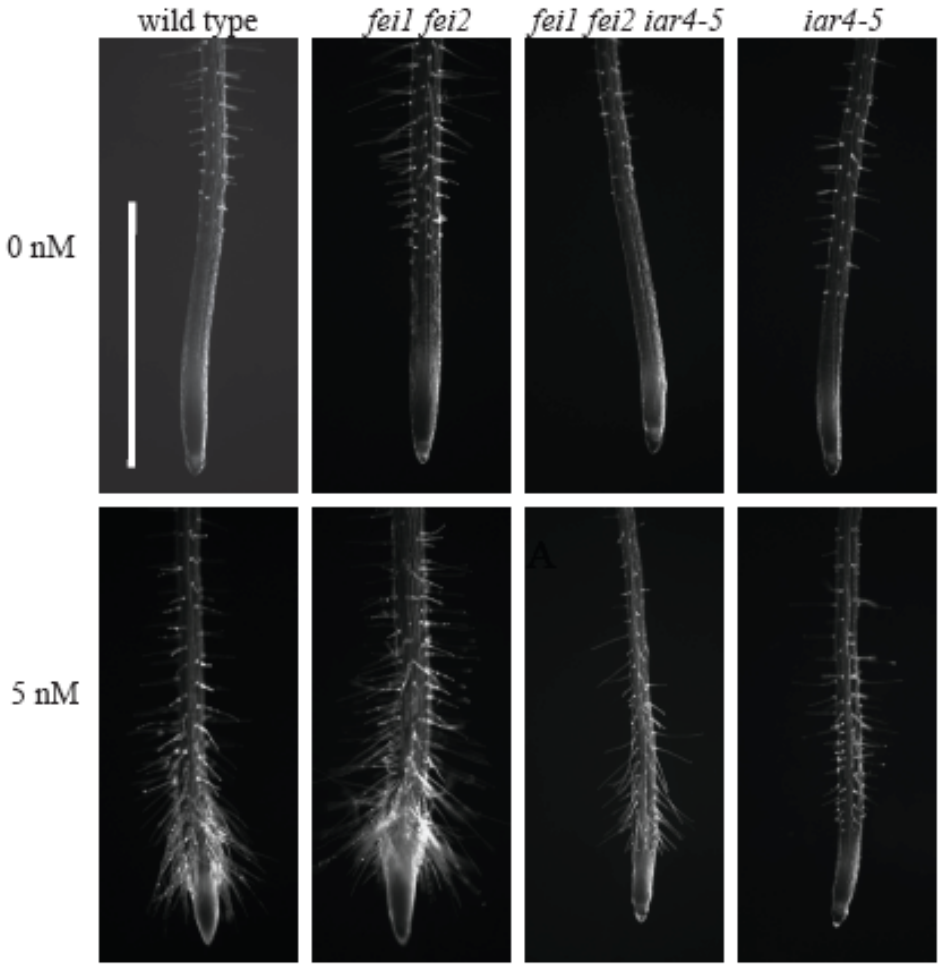


**Figure 2.7 Mutations in *IAR4* confer resistance to isoxaben.**

Root tips of indicated seedlings in response to isoxaben. Seedlings were germinated and grown for 5 days on MS medium with 0% sucrose and transferred to 0 nM of 5 nM isoxaben for twenty-four hours.

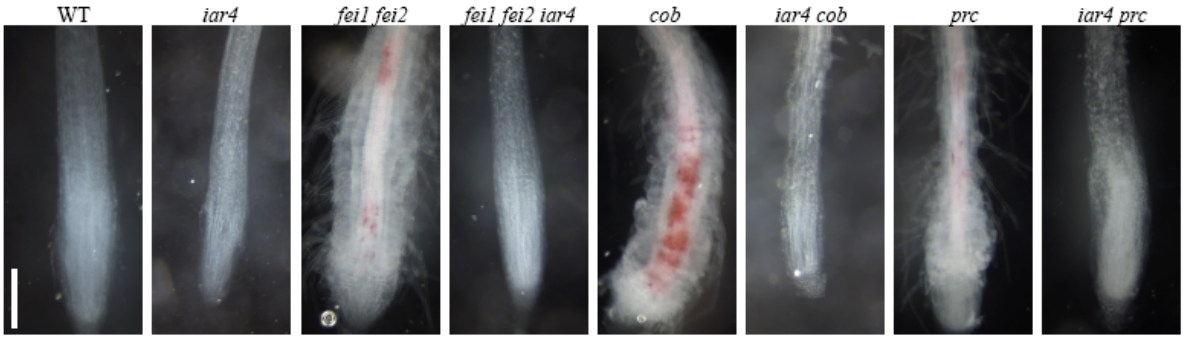


**Figure 2.8 *iar4* suppresses lignin accumulation in cell wall mutants.**

Phloroglucinol stain for lignin (red) accumulation in root tips of seedlings grown on 4.5% sucrose for 2 weeks. Bar = 1mm.

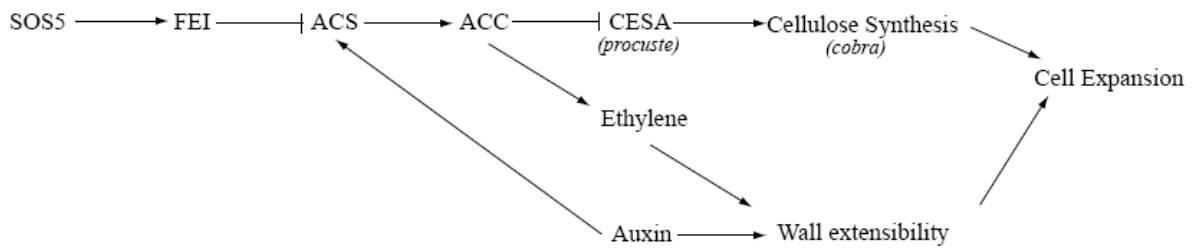


**Figure 2.9 Model of the FEI pathway.**

Hypothetical model depicting the role of both auxin and the FEI pathway in regulating cell expansion. See text for additional details.

# CHAPTER 3

## Further Characterization of the FEI Pathway

The FEI RLKs act together with the extracellular arabinogalactan protein, SOS5, and ACC synthase to promote cellulose biosynthesis and regulate cell expansion in the Arabidopsis root. To determine how this novel signaling pathway regulates cell wall synthesis, we sought to further characterize known elements and uncover additional components of the FEI pathway. In a screen for suppression of the short, swollen root phenotype of *fei1 fei2,* we have isolated several mutants in which growth anisotropy is restored. Of these, we have identified and characterized a semi-dominant allele of one of the genes corresponding to a strong suppressor of *fei1 fei2*, *shou4,* and have mapped the gene corresponding to a separate, loss of function mutation in another suppressor, *shou3,* to the upper arm of chromosome 1. Furthermore, we have confirmed the previously identified interaction between FEI and ACS *in planta* and identified receptor-like kinases that are both co-expressed with FEI1 and FEI2 and that interact with ACS as prospective FEI interacting proteins. To address the mechanism by which the FEI RLKs regulate cellulose biosynthesis, we also examined the relative abundance of CESA protein, and found subtle differences between *fei1 fei2* and wild type. Our studies expand our current

understanding of the FEI pathway and set the stage for future studies by defining an effective approach to mapping suppressors of *fei1 fei2.*

## INTRODUCTION

The cell wall is essential for plant growth and development. It plays an important role in providing structural support, protection against pathogen attack, and in determining the extent and direction to which cells can expand. Despite the importance of the cell wall, our understanding of the molecular mechanisms that regulate its synthesis and control its function remain poorly understood. The identification of the FEI RLKs in Arabidopsis as positive regulators of cellulose biosynthesis provides an opportunity to explore the signaling processes that underlie the biosynthesis of this polymer and as a result, how cell wall function is regulated in plants.

Previous studies on FEI1 and FEI2 suggest a role for the extracellular arabinogalactan protein, SALT OVERLY SESITIVE 5 (SOS5), and ACC synthase in the FEI pathway. When grown under the non-permissive conditions such as high salt or sucrose, s*os5* mutants resemble *fei1 fei2*. Triple *sos5 fei1 fei2* mutants are phenotypically indistinguishable from their parents, indicating that s*os5* likely acts in a linear pathway with FEI to regulate cellulose biosynthesis. In addition to SOS5, members of the type-2 subfamily of ACC synthase interact with both FEI1 and FEI2 in a yeast-two-hybrid assay and this interaction may be physiologically relevant as inhibiting ACC suppresses a loss of growth anisotropy and restores cellulose biosynthesis in *fei1 fei2* (Xu *et al*. 2008). Together, these data suggest that ACC, but

not ethylene, may act as a signal in the FEI/SOS5 pathway to regulate cell wall synthesis. The characterization of ACC synthase and SOS5 has made a significant contribution to our understanding of how the FEI proteins may regulate cell wall synthesis and has set the stage for future research.

In the primary plant cell wall, cellulose biosynthesis requires the assembly of three different CESA proteins into a hexameric rosette at the plasma membrane. Here, CESA1 and CESA3 are required for cellulose biosynthesis. CESA2, CESA5, CESA6, and CESA9 also function in primary cell wall synthesis and are partially redundant with each other throughout different stages of growth (Desprez *et al.* 2007). Of these, CESA1, CESA3, and CESA6 are highly expressed in the root and mutations in any one of them disrupt anisotropic cell expansion (Endler and Persson, 2011). As expected for genes that encode proteins that function in the same complex, CESA transcript levels as well as the abundance of at least CESA3 and CESA6 protein is co-regulated (Desprez *et al.* 2007). In addition, CESA proteins are post-translationally regulated via phosphorylation, which has been shown to target them for degradation and alter the bidirectional mobility of the cellulose synthase complex (Taylor *et al.* 2007; Chen *et al.* 2010).

Here we sought to uncover the molecular mechanism underlying FEI's regulation of cellulose synthesis through the identification and characterization of additional components of the FEI pathway by isolating suppressors of *fei1 fei2* and mapping the corresponding genes. In addition, we identify three different LRR-RLKs as potential FEI interacting proteins and further characterize known components of

the FEI pathway by exploring the FEI-ACS interaction *in planta* and assess CESA stability in *fei1 fei2.*

## RESULTS

### Isolation and characterization of suppressors of the *fei1 fei2* mutant

We screened approximately 200,000 $M_2$ individuals from 145 different pools of ethyl methanesulfonate mutagenized *fei1 fei2* seed for suppression of the short, swollen root phenotype of *fei1 fei2* seedlings in the presence of high sucrose. A total of 67 $M_2$ individual suppressors, designated as *shou* mutants (after the Chinese word for thin), were initially isolated and re-screened in the $M_3$ generation. Of these, twenty-two *fei1 fei2* suppressors did not re-test, thirty-five proved to be weak suppressors, and ten displayed strong suppression of the *fei1 fei2* phenotype (Fig 3.1 and Table 2.2). The weak suppressors displayed partial restoration of root elongation or incomplete suppression of swelling in the root tip, whereas the root tips of the strong suppressors elongated comparable to wild type and did not swell when grown on MS agar supplemented with 4.5% sucrose.

To determine the nature of the suppressor mutations, each suppressor was backcrossed to the parental *fei1 fei2* line. The $F_1$ progeny from each backcross were grown under the non-permissive conditions and root elongation quantified and compared to *fei1 fei2*. As a single copy of a loss of function allele would not be sufficient for suppression of the *fei1 fei2* phenotype, this analysis allowed us to determine whether each of the suppressor mutations were recessive or dominant. Interestingly, one copy of the allele corresponding to four (80C, 88A, 97A, 120A) of

the eight strong *fei1 fei2* suppressors was sufficient for partial suppression of growth isotropy (Fig 3.1). This data indicated that several of the strong suppressors contained semi-dominant mutations. The root length of $F_1$ progeny of backcrosses with three suppressors (145A, 124B, 83A) displayed a non-suppressed phenotype, consistent with these alleles being loss of function, recessive mutations (Fig 3.1). Although it was a strong suppressor, 127A, was left out of this analysis.

To further characterize the *fei1 fei2* suppressors, we grew them in the presence of various concentrations of the synthetic auxin analog, 2,4-dichlorophenoxyacetic acid (2,4-D) but did not observe a change in sensitivity to this compound (Fig 3.2). In addition, we examined whether the *shou* mutations could suppress the increased width of the hypocotyls of etiolated *fei1 fei2* seedlings. We observed partial suppression of the hypocotyl phenotype for each of the strong suppressors examined (Fig 3.3).

**Bulk mapping suppressors of *fei1 fei2***

We combined a map-based cloning approach with whole genome sequencing to identify the gene corresponding to the strong semi-dominant suppressor 97A, which we renamed *shou4.* As described previously (see Chapter 2; Materials and Methods), the *fei1* and *fei2* mutations were introgressed into the Landsberg *erecta* (L*er*) ecotype and this plant was crossed to the *fei1 fei2 shou4* suppressor to generate a mapping population for *shou4*. The semi-dominant nature of the *shou4* mutation made confirmation of the *fei1 fei2 shou4* phenotype in the $M_3$ generation of the mapping population essential for the successful identification of homozygous

individuals. Following an assessment of the $M_3$ progeny, we identified a total of eighty-nine $M_2$ seedlings from the mapping population as phenotypically homozygous for *shou4*.

Using a collection of forty-four markers that spanned the five chromosomes of the Arabidopsis genome and the DNA from phenotypically homozygous $M_2$ seedlings, we used a map-based cloning approach to roughly localize *shou4* (Table 2.3). Initially, a pool of twenty individuals was used to map the *shou4* mutation. However, using this relatively large pool of genomic DNA, we failed to link any one of the forty-four markers to the suppressor mutation, suggesting we may be working with a larger number of false positives than expected. To address this, we partitioned the genomic DNA from a total of thirty individuals into five small pools each consisting of six individuals. Instead of one relatively large pool, we examined each of the small pools of genomic DNA for linkage to any one of the forty-four markers. Using this approach, we successfully linked *shou4* to marker 10, which is located 29.49 Mbp down chromosome 1 (Fig 3.4). Consistent with the idea that we were working with a number of false positives, comparison of the genotypes across individuals from a pool that was heterozygous at marker 10 to those of individuals from a pool that was homozygous at marker 10, revealed a relatively large number of contaminants, or false positives, in the pool that was heterozygous. We genotyped each of the eighty-nine individuals originally identified as homozygous by phenotype and found that only forty-seven of the eighty-nine were homozygous by genotype. Our results indicate that there may be modifiers of the *fei1 fei2* phenotype

in the L*er* background and demonstrate that successful identification of homozygous *fei1 fei2* suppressors relies on the genotyping of previously phenotyped individuals.

To confirm the localization of *shou4*, we examined four other markers; ATPase (28.5 Mbp), nga692 (28.841 Mbp), F9K20 (29.622 Mbp), and F516 (30.2 Mbp) (Fig 3.4). Of the forty-seven individuals examined, we found that thirty-three were homozygous for all five markers and fourteen were recombinant for at least one marker. Interestingly, a greater number of recombinants were identified for ATPase and nga692 suggesting *shou4* is located further than 28.841 Mbp down chromosome 1.

In addition to *shou4,* we have used this approach to map a recessive suppressor, 145A, designated *shou3,* and obtained preliminary data that links *shou3* to marker 4 at the top of chromosome 1 (Fig 3.5).

**Whole genome sequencing of the *shou4* suppressor**

Genomic DNA from the forty-seven $M_2$ lines homozygous for marker 10 was pooled and sequenced on a single lane of the Illumina platform using paired end sequencing. Approximately 200 million paired end reads were generated and mapped to the TAIR9 release of the Columbia Arabidopsis genome. We obtained even coverage of the genome and an average mean depth of 50-60 fold. Using the samtools pileup software, an unfiltered, raw file of single nucleotide polymorphisms was produced for further analysis.

We used the web application Next Generation Mapping (NGM) to identify *shou4.* NGM initially bins and plots SNP frequencies in 250 kb intervals across the Arabidopisis genome. Regions lacking in SNPs correspond to non-recombinant blocks created by linkage to the causative mutation (Austin *et al*. 2011). The background *fei1* and *fei2* mutations, which were originally created by T-DNA insertions, localize to the upper arm of chromosome 1 and the lower arm of chromosome 2 respectively (Xu *et al.* 2008). When *fei1* and *fei2* were introgressed into the Landsberg *erecta* parent, they created non-recombinant blocks in the genome that were identified in the SNP plots created using NGM (Fig 3.6). An additional non-recombinant region lacking in SNPs was identified near the end of chromosome 1 (Fig 3.6). We examined this non-recombinant region specifically for SNPs between the *shou4* suppressor and the annotated reference genome. NGM uses a 'discordant chastity' ($Ch_D$) value to quantify the proportion of reads at a polymorphic site that differ from the reference genome (Austin *et al.* 2011). A $Ch_D$ of 0 is expected for all positions where a base matches the reference genome whereas a $Ch_D$ of 1.0 is expected for all positions in which the base differs from the reference genome. Thus the $Ch_D$ is a measure of the likelihood that a SNP corresponds to the causative mutation. We set the $Ch_D$ to 0.8 to identify SNPs with a high likelihood that they corresponded to the causative mutation. We did not apply any of the program's filters to include all mutations in our analysis. Consistent with results from the bulk segregant analysis, we identified a G/C→A/T transition 29.65 Mbp down chromosome 1 in a cryptic splice site of AT1G78880 (Fig 3.7). Sanger sequencing of AT1G78880 in both the *fei1 fei2* parent and in the *fei1 fei2 shou4* suppressor

confirmed the presence of the G/C→A/T substitution in the splice site at the junction between the second intron and third exon in the suppressor, but not the *fei1 fei2* parent (Fig 3.7A). Consistent with the fact that *shou4* lies within a splice site, comparison of the *SHOU4* transcripts from the suppressor to wild type revealed alternative splicing of *SHOU4* mRNA in the *fei1 fei2 shou4* mutant (Fig 3.7B).

**Characterization of *SHOU4***

*SHOU4* encodes a transmembrane protein of unknown function (Benschop *et al.* 2007). The lack of literature and data on SHOU4 makes it difficult to determine the role of *SHOU4* in the FEI pathway. However, we are in the initial stages of characterizing the *shou4* mutation as it relates to *fei1 fei2.* As shown previously, mutations in *shou4* partially suppress the hypocotyl phenotype of *fei1 fei2* (Fig. 3.3). In addition to suppression of the hypocotyl phenotype of etiolated *fei1 fei2* seedlings, we assessed whether *shou4* suppressed the *fei1 fei2* seed mucilage phenotype. In a developing wild type seed, transverse rays of cellulose anchor the pectin-rich mucilage to the seed surface (Young *et al*. 2008; Blake *et al*; 2006; Dagel *et al,* 2011). In *fei1 fei2*, cellulose synthesis is disrupted which results in the lack of rays in the mutant seed (Harpaz-Saad *et al*. 2011). In order to determine whether *shou4* restores the formation of rays of cellulose, we stained *fei1 fei2 shou4* seeds with calcofluor and pontamine fast scarlet (S4B) for cellulose. We observed no significant difference between *fei1 fei2* and *fei1 fei2 shou4* (Fig 3.8) indicating that *shou4* suppresses some, but not all *fei1 fei2* phenotypes.

**Further characterization of known elements in the FEI pathway**

Previous work from our lab suggests that ACC may act as a signal in the FEI pathway. Type-2 ACS proteins interact directly with the kinase domains of both FEI1 and FEI2 in a yeast-two-hybrid assay (Xu *et al.* 2008). To confirm the FEI-ACS interaction in *planta,* we used bimolecular fluorescence complementation (BiFC). We tested whether FEI1 and FEI2 fluorescently tagged with the C terminal half of YFP (cYFP) interact with ACS5 tagged with the N terminal half of YFP (nYFP). Consistent with findings *in vitro*, we observed reconstitution of YFP in the epidermis of leaves of *Nicotiana benthamiana* transiently expressing ACS5 and either FEI1 or FEI2. Unlike FEI1 and FEI2, the LRR-RLK, HAESA, which controls floral organ abscission, did not interact with ACS suggesting that this interaction is specific to the FEI proteins (Fig 3.9).

The defects in cellulose biosynthesis in *fei1 fei2* suggest that cellulose synthase is either differentially regulated or functionally compromised in the mutant. Microarray data on *fei1 fei2* root tips collected three days after transferred to high sucrose suggests that cellulose synthase is not transcriptionally down-regulated in *fei1 fei2*; however, it is possible that the decrease in cellulose biosynthesis is a result of a change in the post-translational regulation of cellulose synthase. To determine whether the defects in cellulose biosynthesis in the *fei1 fei2* mutant are a consequence of altered levels of cellulose biosynthesis, we examined the abundance of CESA1, CESA3, and CESA6 in *fei1 fei2* under the permissive and non-permissive conditions. We first examined the levels of CESA3 protein at two and three days after transfer to high sucrose. Approximately 48 hours after transfer

to high sucrose, the abundance of CESA3 increased in the root tips of wild type seedlings whereas it remained unaltered in *fei1 fei2* (Fig 3.10). Consistent with this, the abundance of CESA1 and CESA6 did not change in *fei1 fei2* 48 hours after transfer to 4.5% sucrose (Fig 3.11).

**Identification of novel FEI interacting proteins**

In addition to a forward genetics approach, we have performed a co-expression analysis to facilitate the identification of FEI interacting proteins. Often, genes that are expressed simultaneously encode protein products that participate in a shared function. Using multiple co-expression analysis programs such as Virtual Plant, Expression Angler, ATTED-II, Plant Gene Expression Database, and the Arabidopsis Co-expression Tool (ACT), we have identified several RLKs that are co-expressed with FEI1, FEI2, and both FEI1 and FEI2. Although numerous LRR-RLKs were identified, we focused on those identified by at least two or more programs. The LRR-RLK, At1g28040, was identified as co-expressed with FEI1, At2g26730 and At3g08680 as co-expressed with FEI2, and At5g10020 as co-expressed with both FEI1 and FEI2. In addition, At4g08850, named FIL1 (**F**EI-**I**nteracting **L**RR-RLK), was previously identified as a FEI interacting RLK by yeast 2 hybrid (unpublished data). FIL1, like FEI1 and FEI2, contains an intracellular kinase domain and several leucine-rich repeats in its extracellular domain. Interestingly using bimolecular fluorescence complementation (BiFC) we found that the kinase domains of FIL1, At2g26730, and At5g10020 all interact with ACS *in vivo* (Fig. 3.12).

## DISCUSSION

The LRR-RLKs, FEI1 and FEI2 are required for growth anisotropy and promote cell wall synthesis in *Arabidopsis thaliana.* FEI1 and FEI2 interact with members of the type-2 ACC synthase (ACS) subfamily. Using a variety of different web-based tools, we have identified other LRR-RLKs that are co-expressed with the FEI-RLKs. Our results indicate that in addition to FEI1 and FEI2, two other LRR-RLKs (At2g26730 and At5g10020) identified in the co-expression analysis and one identified in a yeast-2-hybrid assay (At4g08680) also interact with ACS *in planta*.

What is the significance of the interactions between ACS and the LRR-RLKs LRR2 (At2g26730), LRR4 (At4g08680), and LRR5 (At5g10020)? One hypothesis is that the interaction between either FIL1 or another RLK identified in the co-expression analysis and FEI is reminiscent of the BAK1/BRI1 interaction. Upon the perception of brassinosteroid, the brassinosteroid receptor and LRR-RLK, BRI1, hetero-oligomerizes with the LRR-RLK, BAK1, to fully activate the brassinosteroid signaling pathway (Chinchilla *et al*. 2009). The FEI proteins may also function via oligomerization with other LRR-RLKs. This hypothesis is supported by results from our lab indicating that the *fei1 fei2* phenotype can be complemented with a kinase-inactive form of either FEI1 or FEI2, suggesting that FEI may dimerize with a functional kinase *in vivo* to activate downstream signaling events (Xu *et al*., 2008). Interestingly, BAK1 has multiple functions. In addition to brassinosteroid signaling, BAK1 is a component of the signaling processes that underlie cell death, light responses, and plant immunity (Chinchilla *et al*. 2009). Hetero-oligomerization of the

FEI proteins would allow them to function as components of a diversity of signaling processes and might explain why a kinase-inactive form of FEI complements the *fei1 fei2* phenotype. Consistent with the finding that additional LRR-RLKs interact with ACS5 *in vivo*, although the FEI proteins themselves do not seem to phosphorylate ACS, oligomerization of FEI into higher order complexes would position a kinase active, FEI-interacting RLK to carry out such a function. Although we were unable to examine RLK dimerization using BiFC, a different approach such as co-immunoprecipitation will be used in future studies to determine whether these proteins interact in the FEI pathway. Furthermore, we have obtained T-DNA knockouts corresponding to each of the RLKs used here. Although single mutants do not appear to have any obvious phenotypes, we are in the process of generating higher order mutants. Future studies will examine the physiological effect of these mutations.

How exactly do the FEI proteins regulate cellulose biosynthesis? We show here that the relative abundance of CESA1 and CESA3, which are required for cellulose biosynthesis, and CESA6, which is partially redundant with CESA2, CESA5, and CESA9, are more abundant in *fei1 fei2* seedlings in the absence of high sucrose and that unlike in the wild type, their abundance does not change upon transfer to high sucrose, suggesting the FEI RLKs may be required for the localization of the CESA proteins to the plasma membrane. Cellulose synthase is trafficked to the plasma membrane and recycled via vesicles derived from the trans Golgi network (Haigler and Brown, 1986; Paredez *et al*. 2006). In addition, osmotic stress and inhibitors of cellulose synthesis cause rapid internalization of cellulose

synthase into a separate, unique set of microtubule–associated intracellular compartments known as MASCs (Crowell *et al*. 2009). If FEI1 and FEI2 are required for the delivery of the CESA complex (CSC) to the plasma membrane, a greater fraction of CSCs may be confined to intracellular compartments in *fei1 fei2* and this could account for the decrease in cellulose biosynthesis in the mutant. Our results provide some evidence in support of this hypothesis. Despite the decrease in cellulose biosynthesis in *fei1 fei2,* CESA protein levels do not differ significantly between the mutant and wild type after transfer to high sucrose. Although we have not yet examined the abundance of CESA2, CESA5, or CESA9, the lack of a *fei1 fei2* phenotype in the absence of high sucrose suggests that one of these proteins may take the place of CESA6 in the CSC in the absence of FEI1 and FEI2. If *CESA* expression is differentially regulated such that the abundance of only CESA6 increases in response to high sucrose and this response is required for cellulose biosynthesis, the defects in cell elongation in *fei1 fei2* would be restricted to growth under the non-permissive conditions.

FEI1 and FEI2 define a novel pathway involved in positively regulating cellulose biosynthesis and therefore the molecular mechanism underlying the regulation of cell wall function by these receptors will likely require the identification of additional components of the FEI pathway. The identification and characterization of the *shou* suppressors of *fei1 fei2* will facilitate our understanding of how this pathway regulates cell wall synthesis. The suppression of *fei1 fei2* and predicted localization of SHOU4 to the plasma membrane makes it a potential component of the FEI pathway; however, its exact function in this pathway remains an open

question. In order to elucidate the function of SHOU4 in the FEI pathway, we must first determine whether *shou4* represents a gain of function or a dominant negative mutation. A gain-of-function allele of *shou4* may promote cellulose synthesis in the absence of the FEIs. On the other hand, if *shou4* is a dominant negative allele, SHOU4 would negatively regulate cellulose biosynthesis in an otherwise wild type background. In a hypothetical model in which SHOU4 acts as a negative regulator of cellulose biosynthesis in the FEI pathway, FEI1 and FEI2 would promote cellulose biosynthesis by repressing SHOU4. Here, SHOU4 would repress cell wall synthesis in *fei1 fei2,* but the loss of both FEI and SHOU4 function in *fei1 fei2 shou4,* would result in wild-type levels of cellulose biosynthesis. We will examine the effect of loss-of-function alleles of *shou4* on *fei1 fei2* to determine which of these models is correct. Future work will also address the patterns of *SHOU4* expression, SHOU4 protein localization, and the relationship between *shou4* and other cell wall mutants. Moreover, whether *shou4* restores cellulose biosynthesis in *fei1 fei2* will be examined to uncover the role that SHOU4 plays in the FEI pathway.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* plants were grown as described previously (Chapter 2; Materials and Methods). Unless otherwise noted, seedlings were germinated and grown on MS agar supplemented with 4.5% sucrose. *Nicotiana benthamiana* plants were germinated and grown on soil on a 16/8h day/night cycle for 4-6 weeks prior to infiltration. For the analysis of root elongation, seeds were germinated on 4.5%

sucrose and total root elongation was measured after 14 days. For the hypocotyl elongation assay, seedlings were exposed to light for 3 hours and grown for 4 days in the dark on MS agar supplemented with 1% sucrose. The width of each hypocotyl was measured 1 mm from the hook of an etiolated seedling.

**Protein extraction and western blot analysis**

For the sample preparation, root tips were harvested after 48 hours on high sucrose and ground with a mortar and pestle in liquid nitrogen into a fine powder. Tissue was resuspended to 50 mg/mL with 2x SDS-PAGE extraction buffer, vortexed, and immediately boiled for 5 minutes. Following a one-minute centrifugation, the supernatant was removed and the pellet discarded. A total of 10 μl of sample was loaded into an SDS-PAGE gel (10% resoling gel and 5% stacking gel) and run at 150V. For the western blot, the gel was transferred at 100V for 1 hour to 0.1 μm nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk in PBST for 1 hour at room temperature. The primary antibody, Anti-AtCesA (courtesy of Dr. Ming Tien and his student, Joseph Hill at Penn State University) was diluted 1:500 in 5% non-fat dry milk in PBST for 1 hour at room temperature. The membrane was washed for 5 minutes for a total of 5 times in 1X PBST. The secondary antibody, anti-rabbit conjugated to alkaline phosphatase, was diluted 1:6,666 and incubated for 1 hour at room temperature. The membrane was washed as again as described and developed immediately following a 5 minute ECL reaction.

**mRNA transcript analysis**

Total RNA was extracted from *fei1 fei2 shou4* and wild type seedlings using a Qiagen RNeasy Mini Kit and treated with TURBO DNAse to remove residual, contaminating DNA. cDNA was synthesized from mRNA using SuperScript III reverse transcriptase (Invitrogen). Amplification of *SHOU4* was performed using the oligomers AT1G78880 2.2F

(5'-GGTCCTCTAGACTATTCTGGTTCG-3') and AT1G78880 6R

(5'-TAATGAAGCCGAAGTGGAGC-3').

**Bimolecular fluorescence complementation**

*FEI1, FEI2, ACS5,* (kindly provided by Shouling Xu), *HAESA* (kindly provided by Michelle Leslie and Christian Burr, University of North Carolina), N2G26730 (ABRC), N3G08680ZE_K (ABRC), N4G08850ZE_K (ABRC), and N5G10020 (ABRC) were cloned into the destination vectors pBAT-YN and pBAT-YC using Gateway technology to generate nYFP and cYFP fusions. Destination clones were transformed subsequently into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* carrying clones of interest and the gene silencing inhibitor p19 (courtesy of Nguyen Phan, University of North Carolina) were grown initially overnight at 28° in 5 ml LB medium with 50 μg/mL rifampicin, 50 μg/mL gentamycin, and 100 μg/mL spectinomycin. An aliquoit from the overnight growth was inoculated into 50mL of media containing the indicated antibiotics and 20 μM acetosyringone and grown overnight. Bacterial cultures were harvested by centrifugation and

resuspended to an $OD_{600}$ 0.8 in 10 mM $MgCl_2$ with 10 mM MES and 200 μM Acetosyringone (3,5-dimethoxy-acetophenone; Sigma-Aldrich D134406) and incubated for 4-6 hours. For the transformation, 3 ml of each construct of interest were combined with 3ml of p19. Using a syringe, approximately 3 ml of culture were infiltrated into the leaves of *Nicotiana benthamiana.*

**Seed Staining**

The calcofluor stain was done as described by Willats *et al.* (2001). Seeds were pre-treated with 50 mM EDTA, stained for 20 min in 25 μg/ml fluorescent brightener 28 (Sigma), washed overnight in water and then visualized using a Zeiss LSM710 confocal microscope equipped with a 405 nm laser diode. Pontamine staining was done as described by (Anderson *et al.* 2010). Seeds were stained for 30 min in 0.01% pontamine fast scarlet S4B (Sigma) following a 90 min pre-hydration, washed for 4 hours in water and then visualized using a Zeiss LSM710 confocal microscope equipped with a 561 laser.

**Microscopy**

Reconstitution of the YFP signal was examined in epidermal cells 2 to 4 d after infiltration. Observations were done using a 40X water immersion objective on a Zeiss LSM710 confocal microscope. Excitation of YFP was accomplished using a 514 nm Argon laser. Images were collected from randomly sampled leaf pieces isolated from infected leaves.

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## TABLES

| Weak Suppressors | | Strong Suppressors |
|---|---|---|
| **Root Length Restored** | **Swelling Restored** | **Root Length and Swelling Restored** |
| 129A | 64A | 80C |
| | 66A | 83A |
| | 67A | 88A |
| | 68A | **97A** |
| | 69A | 120B |
| | 70A | 124B |
| | 73A | 127A |
| | 74B | 145A |
| | 78A | ***SHOU5*** |
| | 85A | ***SHOU2*** |
| | 86B | |
| | 89A | |
| | 95A | |
| | 100A | |
| | 101A | |
| | 103B | |
| | 108A | |
| | 110A | |
| | 116A | |
| | 121B | |
| | 122A | |
| | 126C | |
| | 132A | |
| | 135B | |
| | 138A | |
| | 141A | |
| | 6-2-2A | |
| | 10-1-1A | |
| | 18-1-2A | |
| | 19-4-2A | |
| | 20-1-2A | |
| | 35-1-2A | |
| | 37-1-2A | |
| | ***SHOU1*** | |

**Table 2.2 Phenotypes of *fei1 fei2* suppressors.**
Phenotypes of all strong and weak $M_3$ suppressors isolated in the suppressor screen. *SHOU4* was sequenced in those in red. 97A represents *SHOU4*. *SHOU1, SHOU2,* and *SHOU5* were not sequenced because they were previously identified.

| # | MARKER | CHR | POSITION | COL | LER | FORWARD PRIMERS | REVERSE PRIMERS |
|---|---|---|---|---|---|---|---|
| 1 | NF7G19 | 1 | 2.850 | 197 | >197 | TCGTTCAAAACGATTAGATTG | TTCAAAAATCGTGAGATGAAATG |
| 2 | F20D23 | 1 | 5.889 | 233 | +21 | TTATGCCAACTCATGTGGAAAG | TGTCAAAGCGTCTGGTTCTG |
| 3 | F12K8 | 1 | 7.954 | 168 | +37 | ACCAACACCACAACAAAGAC | CTTTTTCTGTTCTTCCGCTATTC |
| 4 | F13K9-30476 | 1 | 9.744 | 188 | -27 | GGTAGATGCCAATGGAGGAA | TTCACATGTTTCAGGCGAAC |
| 5 | F6N18-69636 | 1 | 11.859 | 164 | -21 | TGCTCGGAAAGTAAAAGTTGG | TGGTTCGGTTGGATTTGTTT |
| 6 | T10P12-20470 | 1 | 16.373 | 223 | -23 | TATTGTGTTCCCACCAAACG | TGTTGGGTTGCTATGCATGT |
| 7 | ciw1 | 1 | 18.367 | 159 | -24 | ACATTTTCTCAATCCTTACTC | GAGAGCTTCTTTATTTGTGAT |
| 8 | NF11P17 | 1 | 22.602 | 209 | <209 | TTTCAGTTTGATGATTTATTCGC | CGCAATCGATTTTATTTAAATCC |
| 9 | F20P5 | 1 | 26.464 | 218 | -38 | GATACGTTCAAAATTAGGGACTTC | TGTATTTTGCTAATTGAGGTTATGC |
| 10 | F3F9-74814 | 1 | 29.493 | 187 | -35 | CGTAACATATCTTCATTCGCCTTT | TTTCTGGTCAACTGAAATCCA |
| 11 | T17M13 | 2 | 0.830 | 336 | -43 | CTGGAGATCATCCAACAAAG | TGCAATGGAATGGGCTGGTC |
| 12 | F15L11-12250 | 2 | 1.830 | 253 | -43 | AGAGGCTGATCGGTCTGAAA | GCGGGTGTTACGATAGAGGA |
| 13 | ciw3 | 2 | 6.409 | 230 | -30 | GAAACTCAATGAAATCCACTT | TGAACTTGTTGTGAGCTTTGA |
| 14 | F26B6 | 2 | 10.000 | 223 | +51 | CTCTATCTGCCCACGAACAAG | GCCATTGCAAAAGAACATCAG |
| 15 | T9D9-46843 | 2 | 12.937 | 272 | -45 | GAATTTCTCAATTTTCAGGACTAACA | CGAGATTGAATGGTGATCCA |
| 16 | T1J8-46260 | 2 | 15.492 | 226 | -26 | CGGGCTGTCCATGAACTATT | AAACCAAATCGAACCAACCA |
| 17 | F4I18-34837 | 2 | 18.899 | 290 | -43 | GACCCGAGGGTTATATGCAA | CCGCAGCCATGCTTATTTTA |
| 18 | F13E7-68449 | 3 | 0.617 | 239 | -30 | TCGACTCCAGTCCAAATGTTC | TACGCGTGTTTCCCCTTTAC |
| 19 | nga162 | 3 | 4.608 | 146 | -18 | GCAATTTGCATCTGAGGAAT | GCTCCTGAGTTTCGGACAGA |
| 20 | MSA6-50838 | 3 | 7.405 | 227 | -41 | CCCTGGCAAGACATAACCAA | CTTCTTGTTTTGCCTCTGTGG |
| 21 | ciw11a | 3 | 9.775 | 192 | +50 | GTTTTTTCTAATCCCCGAGTTGAG | GAAGAAATTCCTAAAGCATTC |
| 22 | T13B17-321 | 3 | 11.329 | 202 | -48 | TTTAACAGATTTTAGGAAAACAAATCA | TTGGTAAACAAACCATCACCTTT |
| 23 | T4P3-6097 | 3 | 14.137 | 193 | -25 | CACATTCTCGAGGTGCACTG | GTTACCGCACAAGAGGTCGT |
| 24 | F13I12-58714v2 | 3 | 17.350 | 232 | -26 | TGGACCCAAGTCTTTGGATT | CCCTCGTTTCTCTTTCTCGTT |
| 25 | ciw4 | 3 | 18.901 | 189 | +26 | GTTCATTAAACTTGCGTGTGT | TACGGTCAGATTGAGTGATTC |
| 26 | F27K19 | 3 | 20.820 | 237 | +44 | TGCTTTTGAAGAGATGGTTATTAGG | CCCCATTTCACTTATCATTGG |
| 27 | T20O10-24167 | 3 | 23.292 | 215 | -25 | GGGGAAACAGATAAGGAAGCA | CGCTGTAGCAAACGTGGTAA |
| 28 | ciw5 | 4 | 0.738 | 164 | -20 | GGTTAAAAATTAGGGTTACGA | AGATTTACGTGGAAGCAAT |
| 29 | C17L7-38326 | 4 | 2.683 | 217 | -25 | TGAAGCGGTTTCAAGATTGTT | CGCAAACTATTTGCCATAGTCA |
| 30 | T12G13-90376 | 4 | 5.253 | 168 | -26 | CGATTTCGGTTCGCATCTA | ACCCGAACCCAAAACTAAAAA |
| 31 | T26M18 | 4 | 7.157 | 330 | -59 | ACATTAGCGGAGGCCACTTC | GGGCAAAAGCTTCCAGTAC |
| 32 | F28A21-47297 | 4 | 10.288 | 207 | -29 | GCAATAAAAGAAAGGGGGAAA | TGACAATCACGGATACAAAAGC |
| 33 | F26K10 | 4 | 13.991 | 180 | +25 | AGAGAGCACGATGCCTGATAG | AATGCTTCAGCGATTGAGAAC |
| 34 | F23E13 | 4 | 17.148 | 264 | -39 | TGACCGTTGAAAGTGTTGTTG | GCCCGAGAAGCCTGATAG |
| 35 | T5J17-51431 | 4 | 18.513 | 193 | -19 | GCACACAGAGATATCGAAAAATCA | GCGTTTGATTTCAAATTCGTT |
| 36 | MOJB | 5 | 2.190 | 178 | -20 | GAAGATGAAAGATTTTAGGAGGAC | GTTTGTAGGAGAAGGGGACAAG |
| 37 | F5E19-2891 | 5 | 5.469 | 192 | -17 | GACTAAACTGGCATTAAGTTCTGAG | TCGTAGTTCGTACCTGTGCCTA |
| 38 | MRO11-71713 | 5 | 8.013 | 100 | +35 | CGTTATGTAATAGTCATCACGTTTTTG | TTCACAATTAGAACGCTGAATCAT |
| 39 | nga76 | 5 | 10.419 | 220 | +80 | AGGCATGGGAGACATTTACG | GGAGAAAATGTCACTCTCCACC |
| 40 | phyC.3 | 5 | 14.025 | 211 | +15 | AAACTCGAGAGTTTTGTCTAG | CTCAGAGAATTCCCAGAAAAATC |
| 41 | MFO20-693 | 5 | 17.059 | 228 | -42 | AACTATGTTTGGCATTTAAAGGTT | AGCAAATGGAACCGAAACAA |
| 42 | K9P8 | 5 | 20.312 | 284 | -33 | TTATGGGTTTCTCAGAGTTTCTCAC | TTGTATGCGTTTGCTTTTTCC |
| 43 | MBG8-3386 | 5 | 22.262 | 197 | -39 | TGAACTGGATCAGCTTTACTTGA | AGGTGCACGAGATGGTTCAT |
| 44 | MQB2 | 5 | 25.208 | 252 | -21 | CTTTGATAGTAACCTTTTTCAAACCA | TGCCATTTATTTGGTCAACAC |

**Table 2.3 Mapping primers used to clone suppressors of *fei1 fei2*.**

## FIGURES

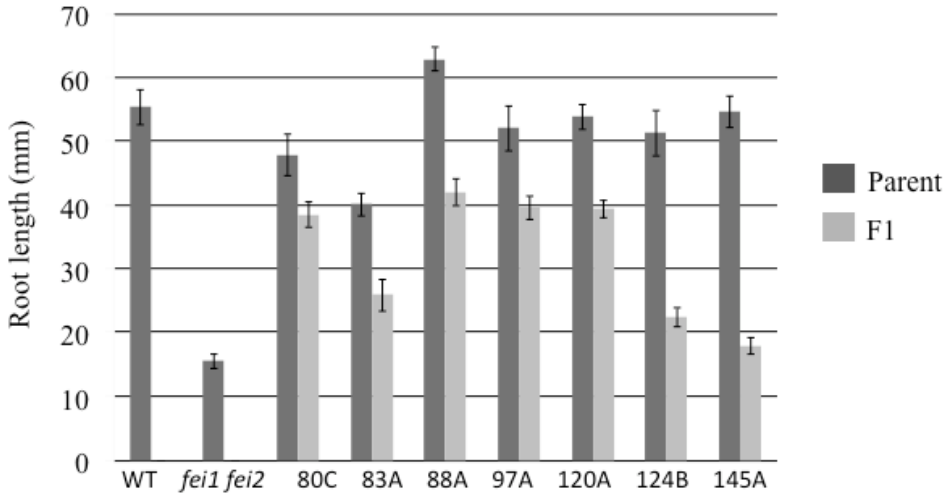


**Figure 3.1 Root elongation of *fei1 fei2* suppressors.**

Dark grey bars indicate root lengths of WT, *fei1 fei2,* and *shou* suppressors. Light grey bars represent root lengths of F1 progeny obtained from a backcross between the *shou* suppressors and *fei1 fei2.*Error bars ±SE.

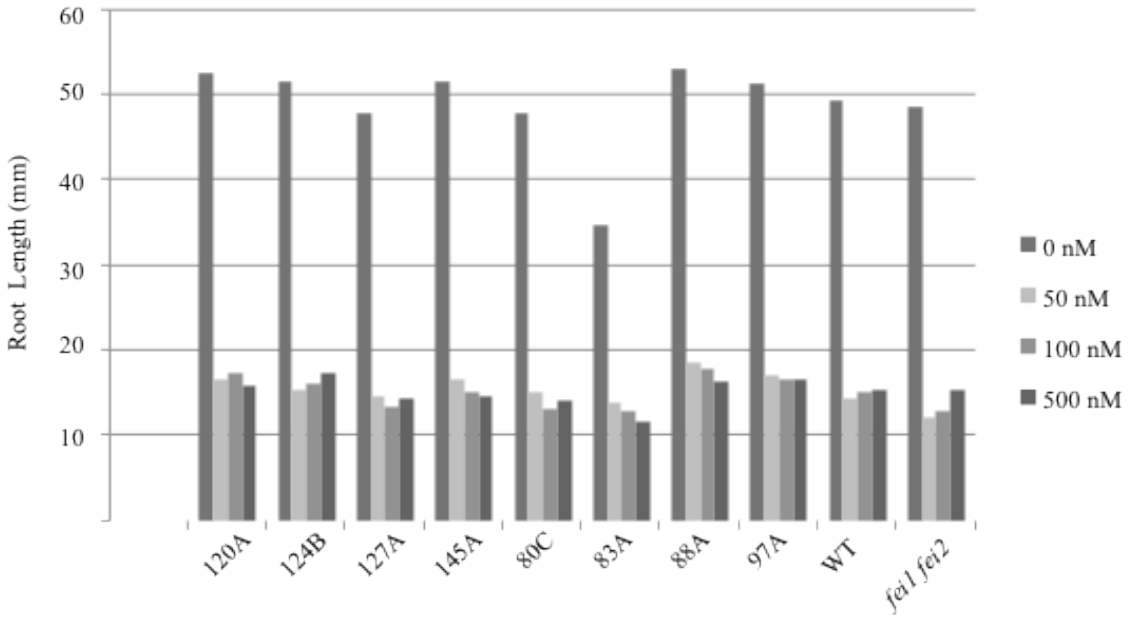


**Figure 3.2 Total root elongation of *fei1 fei2* suppressors in the presence of 2,4D.**

Seedlings were transferred after five days to the indicated treatment and grown for approximately three weeks.

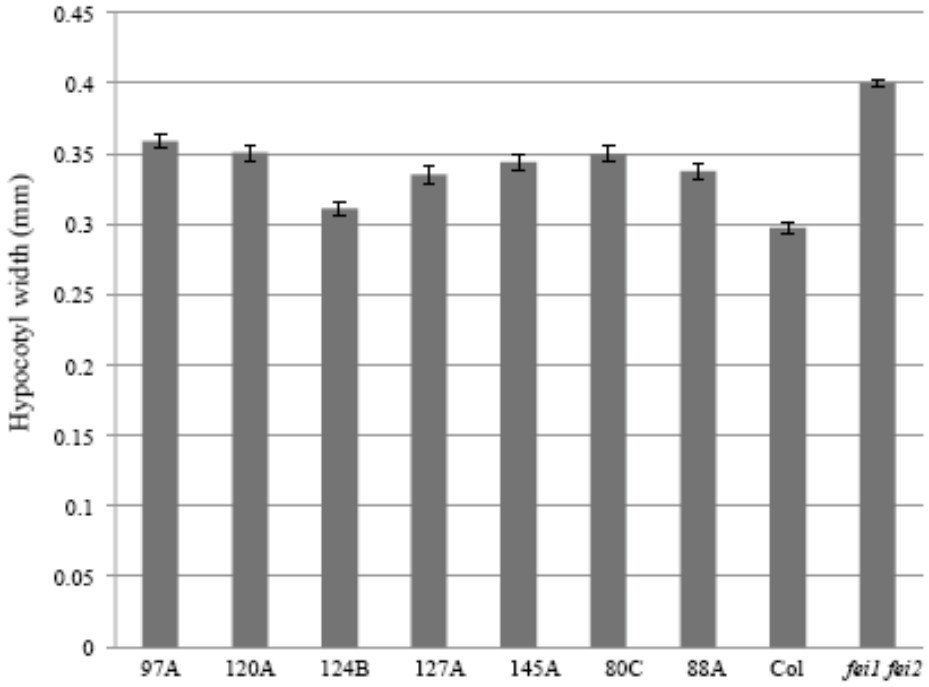


**Figure 3.3 Quantification of hypocotyls widths of *fei1 fei2* seedlings.**

Average widths of hypocotyls of four day old etiolated seedlings. Each suppressor shows partial suppression of the *fei1 fei2* hypocotyl phenotype. Error bars = ± SE.

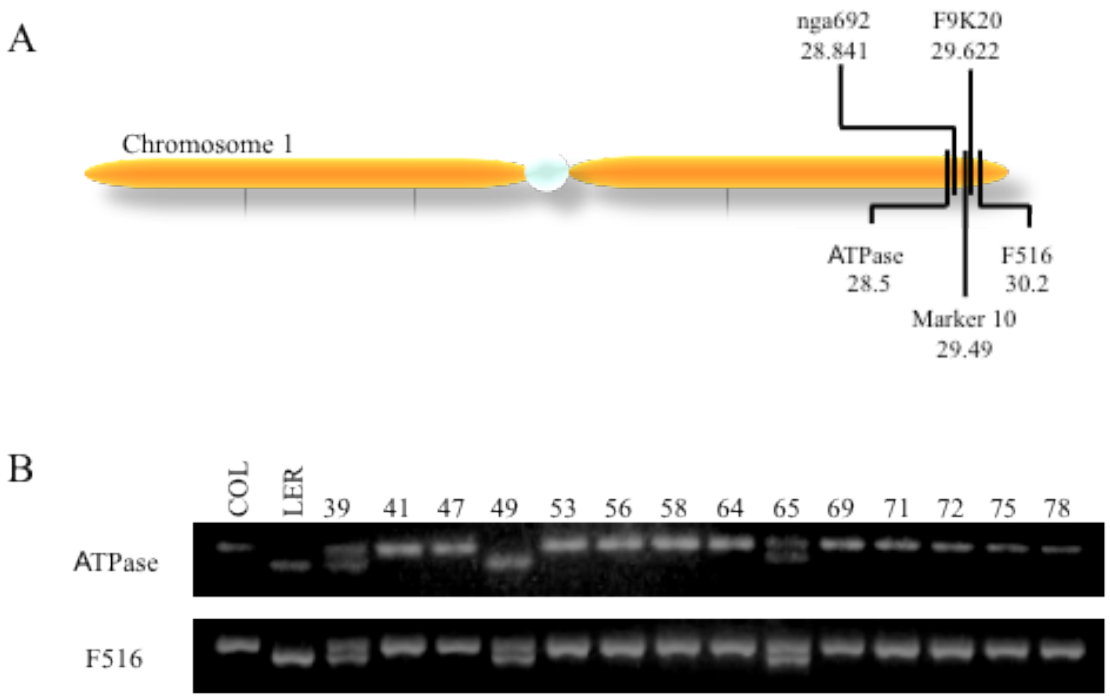


**Figure 3.4 *SHOU4* maps to the bottom arm of chromosome 1. (A)** Cartoon of chromosome 1 and each of the five markers used to localize *SHOU4*. **(B)** Genotyping individual $F_2$ plants from the mapping population indicates that markers ATPase and F516 are linked to the *shou4* mutation. With the exception of 39, 49, and 65, the DNA from all individuals shown was used for further analysis.

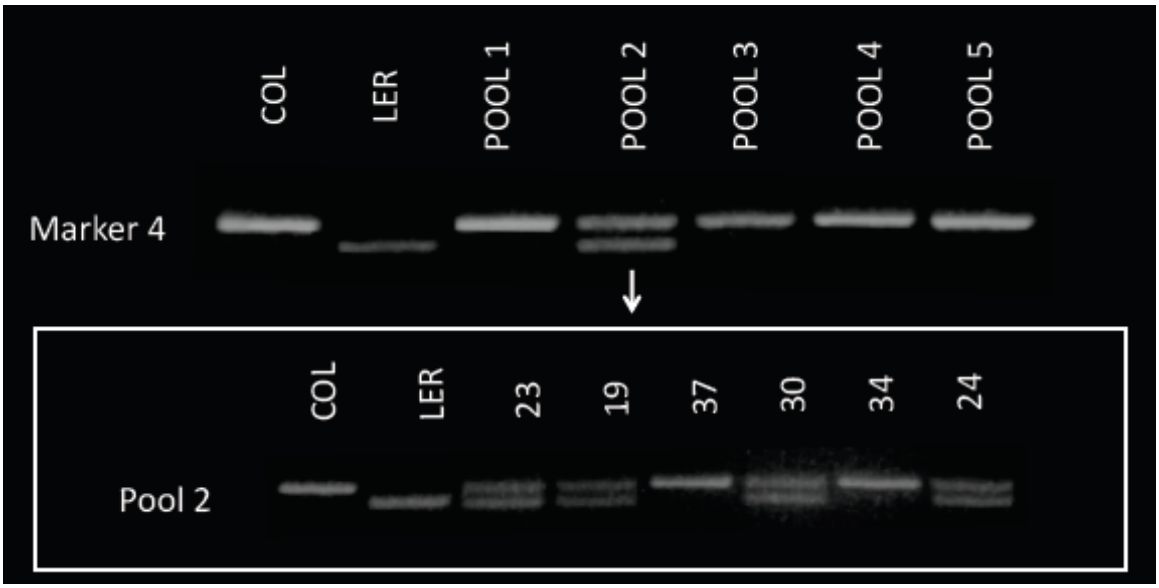


**Figure 3.5 *SHOU3* maps to the top arm of chromosome 1.**

The *shou3* mutation is located approximately 9.744 Mbp down chromosome 1. Top: Pools 1, 3, 4, and 5 show a band specific to Col. Pool 2 is heterozygous for marker 4. Bottom: Genotypes of individual plants from pool 2. Unlike pools 1, 3, 4, 5, pool 2 contained several false positives suggesting that this marker is linked to *shou3.*

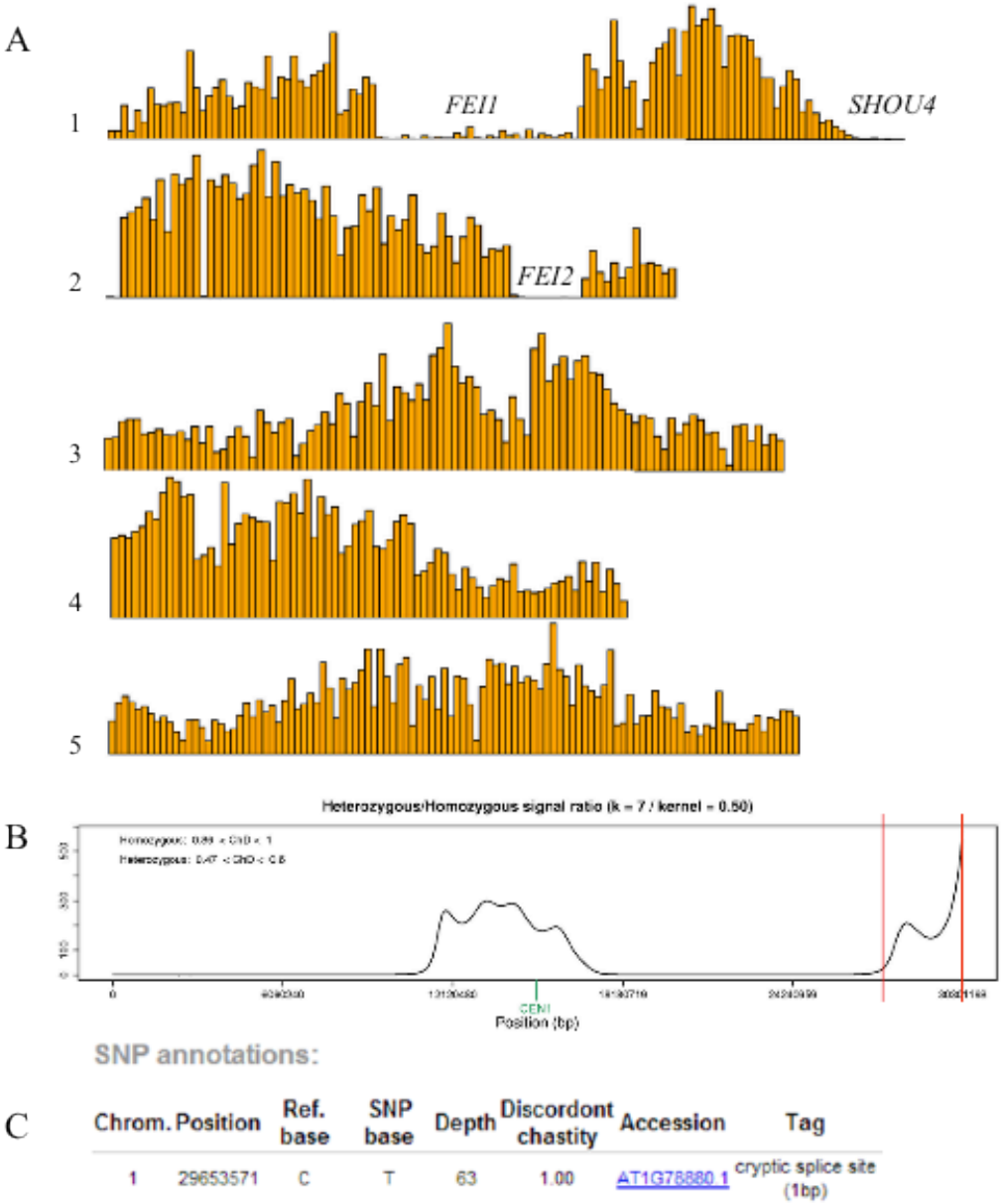


**Figure 3.6 Whole genome sequencing of *fei1 fei2 shou4.***

**(A)** SNP frequencies plotted as a function of chromosome position in *fei1 fei2 shou4.* Deserts on chromosomes 1 and 2 correspond to non-recombinant regions.

**(B)** Dischordant chastity analysis identifies a candidate SNP within the non-recombinant region at the bottom of chromosome 1 in At1g78880 **(C).**

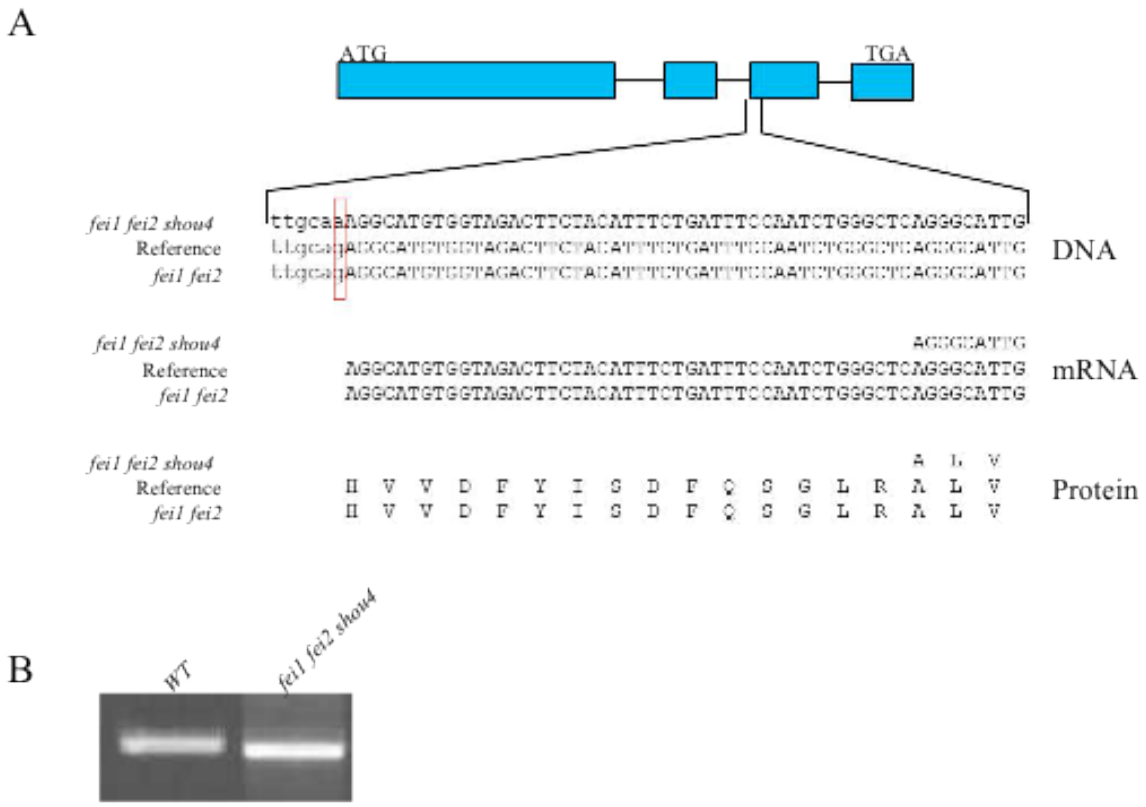


**Figure 3.7 The *shou4* mutation leads to a 45 bp or 15 amino acid in frame deletion. (A)** *shou4* is not present in the *fei1 fei2* parent and leads to a 45 bp deletion in the third exon at a splice site with the second intron. **(B)** Comparison of the *SHOU4* mRNA transcript from WT and *fei1 fei2 shou4*.

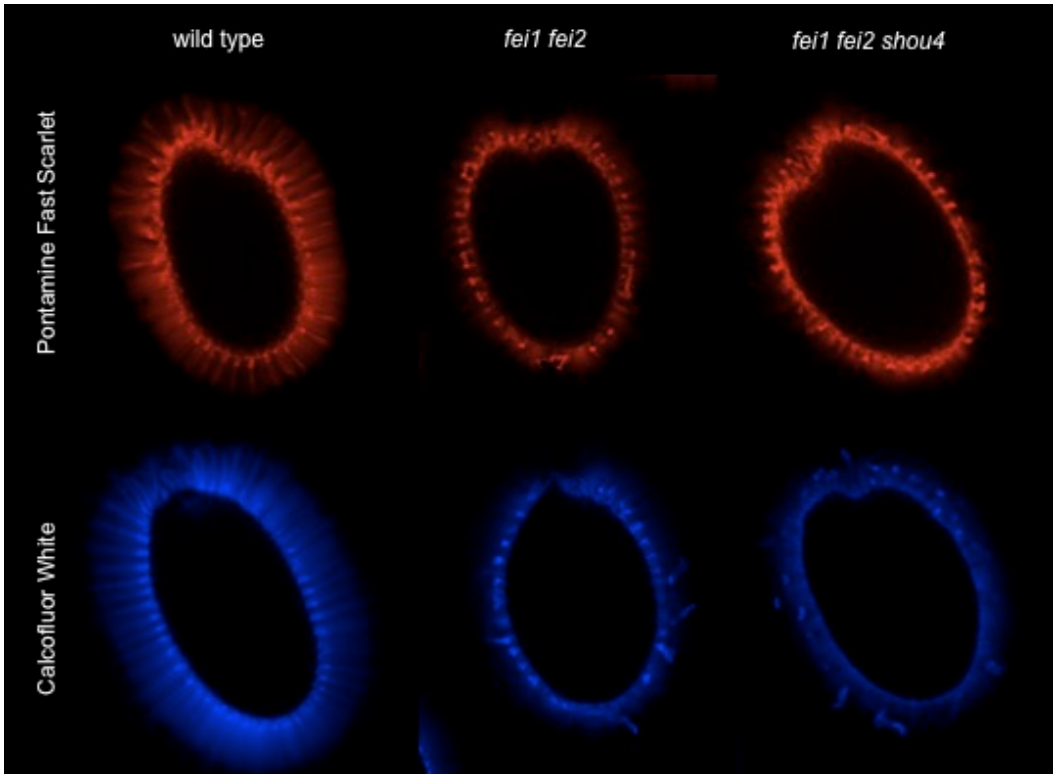


**Figure 3.8 *shou4* does not suppress the seed mucilage phenotype of *fei1 fei2.***

Pontamine Fast Scarlet and calcofluor white stain cellulose rays in seed coat mucilage of seeds from the indicated genotypes.

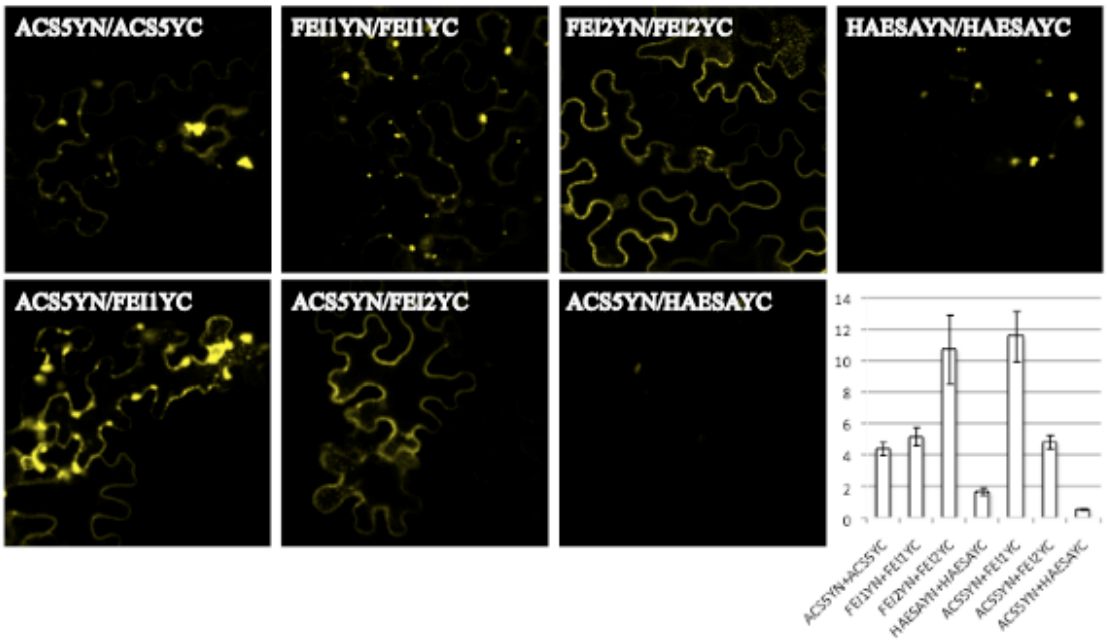


**Figure 3.9 FEI1 and FEI2 interact with ACS *in planta.***

Reconstitution of YFP fluorescence in the epidermal cells of *Nicotiana benthamiana* expressing the indicated fusion proteins.

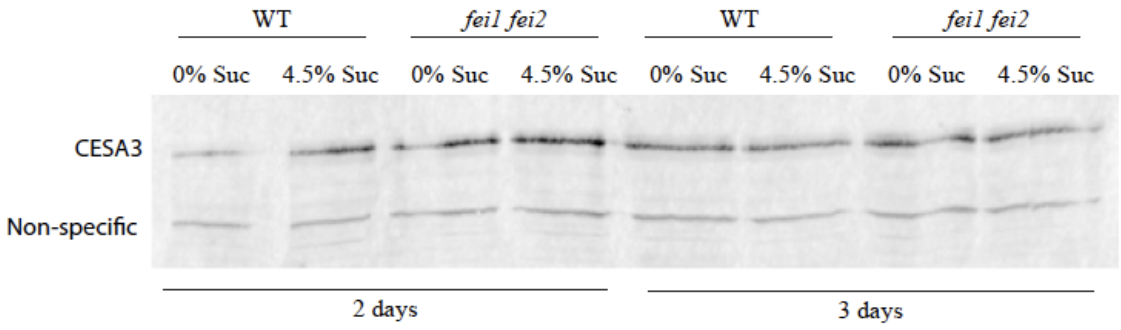


**Figure 3.10 Relative abundance of CESA3.**

Total CESA3 protein exacted from WT and *fei1 fei2* seedlings transferred to high sucrose and grown for two or three days. Note the increase in CESA3 in the root tips of wild type seedlings, but not *fei1 fei2* two days after transfer to high sucrose.

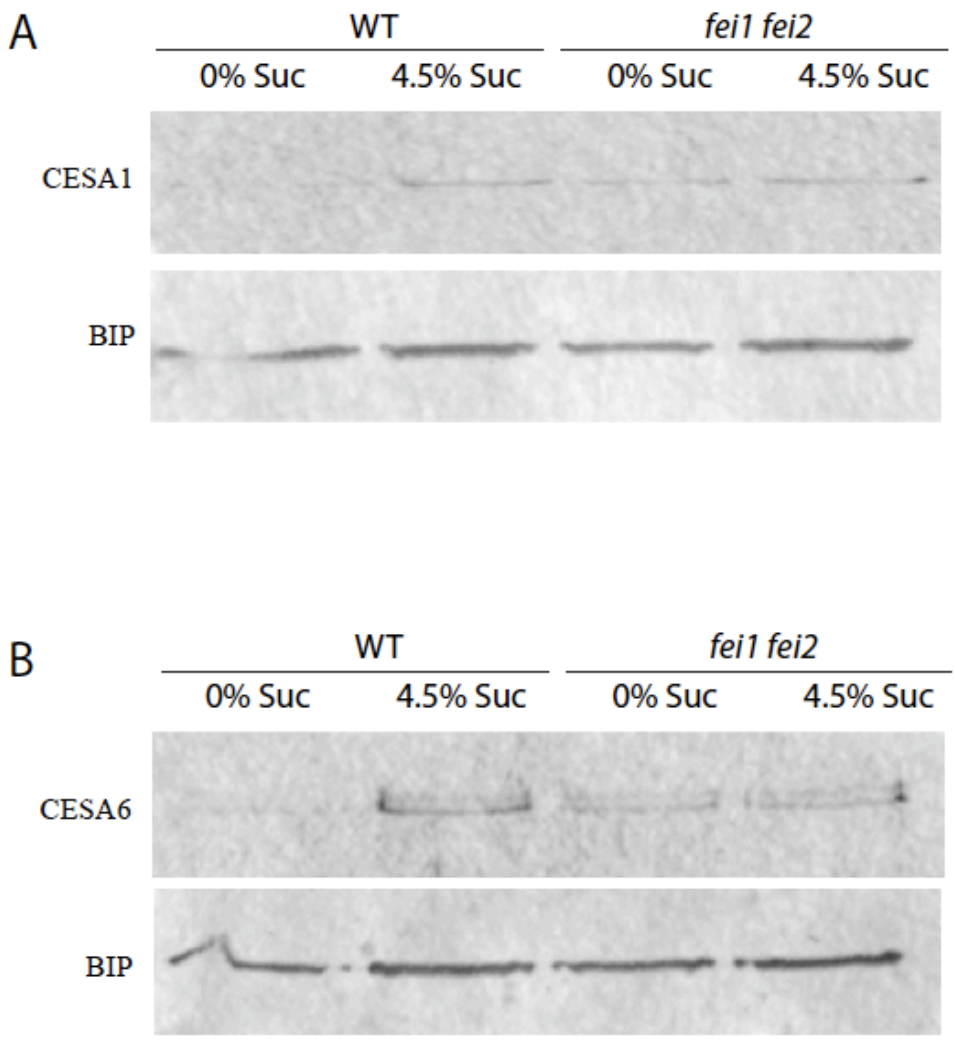


**Figure 3.11 Relative abundance of CESA1 and CESA6.**

Total CESA1 **(A)** or CESA6 **(B)** protein from root tips harvested from WT and *fei1 fei2* seedlings after 2 days growing on 0% or 4.5% sucrose.

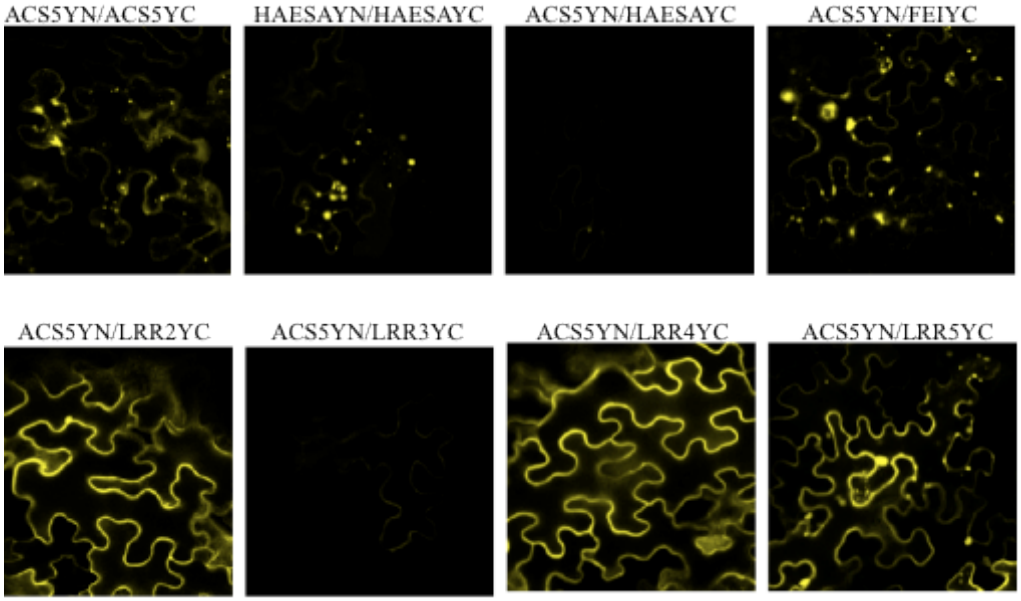


**Figure 3.12 ACS interacts with LRR-RLKs that are co-expressed or interact with the FEI proteins.**

Bimolecular fluorescence complementation showing reconstituted YFP fluorescence in *Nicotiana benthamiana* expressing the indicated fusion constructs.

# CHAPTER 4

# FUTURE DIRECTIONS

## What genes do the *fei1 fei2* suppressors correspond to and what is their role in the FEI pathway?

Elucidation of the molecular mechanism underlying FEIs regulation of cell wall synthesis will require the identification of the genes that correspond to additional suppressors of *fei1 fei2,* which in most cases will precede their characterization as it is difficult to follow the suppressor mutations in the absence of a molecular marker. We have generated mapping populations for each of the strong *fei1 fei2* suppressors and have refined the mapping strategy to facilitate the isolation of novel components of the FEI pathway in future studies. Once the *SHOU* genes have been cloned, their characterization will depend largely on their identity. However, we will examine gene expression patterns, localize the SHOU protein, assess cellulose biosynthesis, and perform a genetic analysis between the suppressor and known cell wall mutants (*prc*, *cob*, *sos5*, etc) for each of the suppressors. In addition, it will be interesting to determine the role of the *SHOU* genes in an otherwise wild-type background. The *shou* mutants will be crossed back to wild type to generate a population of plants segregating for the *fei1, fei2,* and *shou* mutations and a plant homozygous for *shou* selected

for. We will also determine the physiological effects of null alleles, which we will likely be able to obtain from the publicly available T-DNA insertional collection.

**How is cellulose synthase function altered in *fei1 fei2*?**

Interestingly, we have found that CESA1, CESA3, and CESA6 protein levels appear to be altered in *fei1 fei2.* Our results indicate that CESA3 protein levels increase in the root tips of wild type seedlings two days after transfer to high sucrose but remain unchanged in *fei1 fei2.* In addition, CESA1 and CESA6 increase in wild-type seedlings in the presence of 4.5% sucrose but not in *fei1 fei2*. These data suggest the possibility that cellulose synthase may be mis-localized in *fei1 fei2*. If FEI1 and FEI2 are required for the localization of the CSC, the ratio of cellulose synthase at the plasma membrane to that of the intracellular compartments may be altered in *fei1 fei2.* Thus future studies will examine the sub-cellular localization of the CSC. The movement of intracellular compartments derived from the endomembrane system differs from that of microtubule associated cellulose synthase compartments, or MASCs (Crowell *et al*. 2009). Thus, if the CSC is mis-localized in *fei1 fei2*, the movement of the intracellular, CSC containing compartments in *fei1 fei2* could be used to determine where exactly the CSC is located within the cells of the mutant. Our lab has obtained transgenic plants expressing both GFP-CESA3 and GFP-CESA6 from Samantha Vernhette's lab. We have generated *fei1 fei2* plants expressing GFP-CESA6 in order to examine the sub-cellular localization of cellulose synthase in *fei1 fei2.* Together with markers corresponding to different compartments of the cell, these lines will allow us to

examine whether the sub-cellular localization of cellulose synthase is altered in *fei1 fei2*.

Phosphorylation of CESA has previously been shown to regulate the bidirectional mobility of cellulose synthase. Specifically, eliminating or mimicking phosphorylation of CESA at distinct sites alters the rate at which the CSCs themselves move through the plasma membrane. Further, these changes are correlated with changes in anisotropic cell expansion (Chen *et al*. 2010). If our results cannot be repeated and there is in fact no obvious change in the sub-cellular localization of CESA, an alternative model is that FEI1 and FEI2 modulate the phosphorylation of CESA. In order to determine whether the FEI proteins are required for the bidirectional mobility of cellulose synthase, *fei1 fei2* plants expressing GFP-CESA6 could be examined for a change in the velocity of the CSCs. If phosphorylation of CESA does pertain to the FEI pathway, it is most likely via the activity of another RLK that acts in a complex with the FEIs because kinase activity is dispensable for FEI function (Xu *et al*. 2008).

**What is the significance of the ACS-FIL1/LRR4 interaction?**

In order to confirm and further explore the interaction between ACS and FIL1, these proteins should be co-immunoprecipitated from Arabidopsis. If FIL acts in a complex with FEI and ACS to regulate cellulose biosynthesis, then FIL should also interact with the FEI proteins. Our previous work has demonstrated that BiFC is not a useful technique for examining RLK interactions. Thus, in order to explore the possibility that FEI oligomerizes with FIL, co-immunoprecipitation may also be used to answer the question of whether the FEI proteins interact with the FIL proteins. To

determine the physiological relevance of the ACS-FIL interaction, the phenotype of a double *fil1 fil2* (a close paralog of *fil1*) mutant should be examined along with a *fei1 fei2 fil1 fil2* quadruple mutant. If FEI acts together with FIL to negatively regulate ACC, disrupting FIL function may lead to a short, swollen root phenotype as seen in the *fei1 fei2* mutant.

**What is the nature of the FEI-ACS interaction?**

We used BiFC to demonstrate that FEI interacts with ACS in *Nicotiana bentamiana*. This work could further be expanded to the Arabidopsis root where we could explore the effect of environmental and developmental signals on the FEI/ACS interaction. The generation of transgenic plants expressing ACS5-nYFP and FEI-cYFP would allow us to explore the effect of high sucrose and high salt as well as whether the FEI-ACS interaction occurs throughout different stages of growth. Most importantly however, genetic disruption of ACS function is needed for strong evidence that ACC acts as a signal in the FEI pathway. I have generated plants homozygous for *fei1 fei2 acs5 acs9* and *acs11* and crossed them to an *acs4 acs5 acs8 acs9* mutant. The $F_2$ progeny from this cross are segregating for each of the type-2 ACS genes, *fei1,* and *fei2.* Inhibitors of ACC function revert the phenotype of *fei1 fei2* (Xu etal., 2008). Once it has been identified, the septuple mutant will be examined for reversion of the *fei1 fei2* phenotype as the suppression of the short, swollen root phenotype would provide strong evidence that ACC acts as a signal in the FEI pathway.

## REFERENCES

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**Xu SL, Rahman A, Baskin TI, Kieber JJ** (2008) Two leucine-rich repeat receptor kinases mediate signaling linking cell wall biosynthesis and ACC synthase in Arabidopsis. Plant Cell **20:** 3065-3079.