Investigating the role of 1-aminocyclopropane 1-carboxylic acid as a signaling molecule in the FEI cellulose synthesis pathway in *Arabidopsis thaliana*

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Abstract

Cellulose is an important structural component of plant cell walls. Recently, two receptor-like kinases, FEI1 and FEI2, were found to regulate cell wall synthesis in *Arabidopsis thaliana*. The *fei1 fei2* double mutant, when grown on high sucrose, has short, swollen roots due to cellulose deficiency. Ethylene, a gaseous plant hormone, is known to inhibit root elongation and cause root growth defects. It was seen that disruption of ethylene biosynthesis, but not of ethylene perception, led to a reversion of the *fei1 fei2* mutant phenotype. 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate ethylene precursor in its biosynthesis pathway. This, along with other biochemical and genetic analyses, suggests that ACC acts as an independent signaling molecule in the FEI pathway in *Arabidopsis thaliana*. ACC is synthesized from *S*-Adenosyl methionine (AdoMet) by ACC synthase (ACS), and is converted to ethylene by ACC oxidase (ACO). Using CRISPR-Cas9, the eight functional *ACS* genes and five functional *ACO* genes were disrupted in separate plant lines, in wild-type and *fei1 fei2* backgrounds, and confirmed using restriction digest and agarose gel electrophoresis. CRISPR-Cas9 gene editing has been successfully used to disrupt all *ACS* and *ACO* genes, but not in the same plant line. Obtained higher-order mutant lines will be used to study the effect of low and high levels of ACC on cell wall perturbations. This can be used to determine potential downstream effectors of ACC and shed light on its non-canonical role as a regulator of cell wall synthesis.

Introduction

Cell walls in plants are made up of various polysaccharides and may have up to three layers. The primary cell wall is a thin, flexible layer that forms while the cell is growing. The secondary cell wall, located between the plasma membrane and the primary cell wall, provides

additional protection to the cell, and rigidity and support to the plant. The middle lamella is an outer cell wall layer that aids in cell adhesion. (Buchanan et. al. 2015). Cellulose is an important structural component of the primary cell wall and consists of linear chains of β (1→4) linked glucose units (Updegraff 1969). Cellulose microfibrils, formed when hydroxyl groups on one chain form hydrogen bonds with oxygen atoms on neighboring chains, confer high tensile strength to the cell wall (Arioli et. al. 1998). The orientation and cross-linking of these microfibrils are key factors in determining the direction and extent of cell expansion. Plants generally grow anisotropically – in a direction-dependent manner – due to the restriction of radial expansion (Baskin 2005). Disruption of cellulose biosynthesis results in a rapid loss of anisotropy and causes cells to grow isotropically – equally in all directions (Desprez et. al. 2002). In particular, perturbation of cell wall integrity in *Arabidopsis* affects the elongation phase of root cells and can result in root swelling (Tsang et. al. 2011).

While several phytohormones affect root growth, the effect of ethylene on elongation is particularly well-characterized (Benkova and Hejatko, 2009). Ethylene is a plant hormone that affects many aspects of plant growth and development. Ethylene biosynthesis is a tightly regulated process that begins with the conversion of the amino acid methionine to S-adenosyl-methionine (AdoMet) by AdoMet synthase. AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). Lastly, ACC is oxidized by ACC oxidase (ACO) to form ethylene (Fig. 1A) (Yoon and Kieber 2013). In *Arabidopsis*, ACS proteins are encoded by eight genes – *ACS2*, *ACS4, ACS5, ACS6*, *ACS7, ACS8, ACS9* and *ACS11* – and ACO proteins are encoded by five genes – *ACO1, ACO2, ACO3, ACO4,* and *ACO5*. ACS proteins can be classified into three families based on differences in their C-terminal domains. Type-1 proteins (ACS1, ACS2, and ACS6) have long C-terminal ends that contain a calcium-

dependent protein kinase (CDPK) phosphorylation site and three mitogen-activated protein kinase (MAPK) sites. Type-2 proteins (ACS4, ACS5, ACS8, ACS9, and ACS11) have only the CDPK site at the C-terminus. Type 3 protein (ACS7) has no predicted phosphorylation site at the C-terminus (Hansen et. al. 2008).

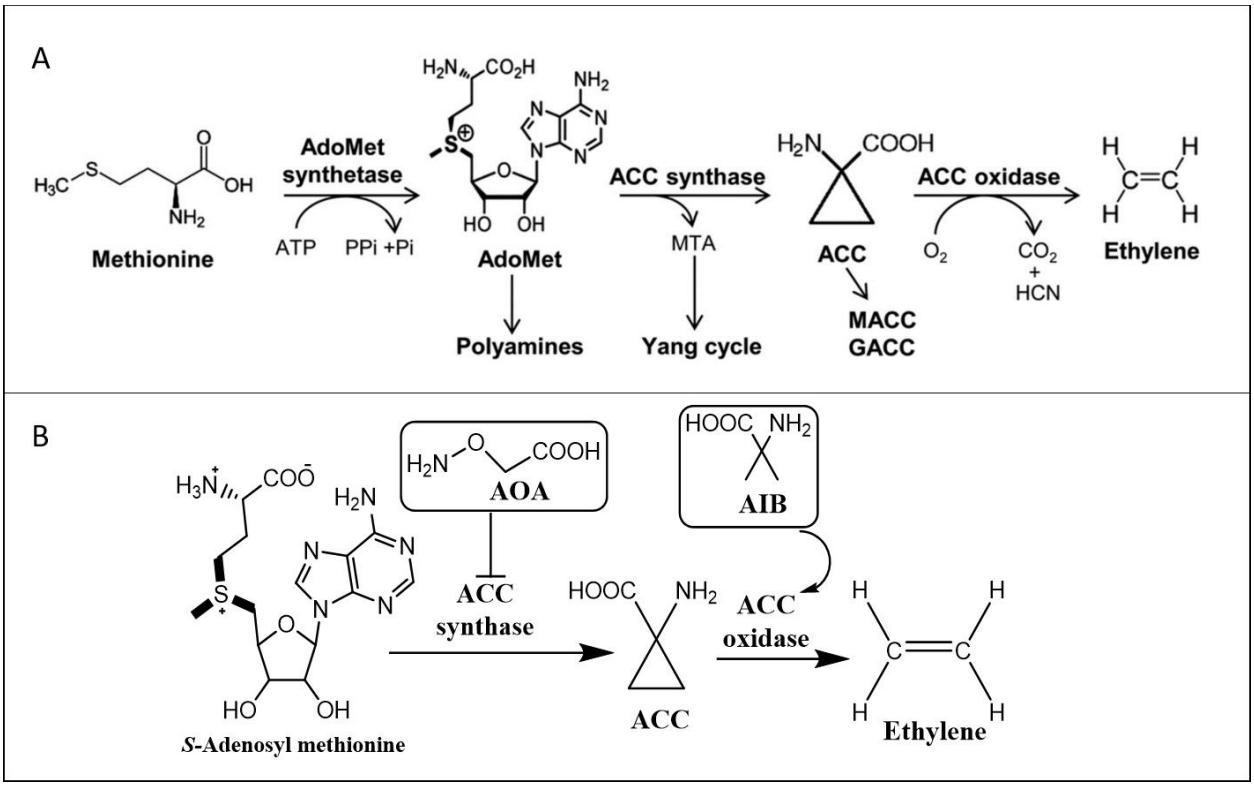

Figure 1: (A) Ethylene biosynthesis pathway. ACC can also be conjugated as malonyl-ACC (MACC) and 1-(γ-1-glutamylamino) cyclopropane-1-carboxylic acid (GACC). (Yoon and Kieber 2013) (B) Action of aminooxyacetic acid (AOA) and α-amino[1-(14)C]isobutyric acid (AIB) in inhibiting ethylene biosynthesis(Broun and Mayak 1981;Saftner and Baker 1987).

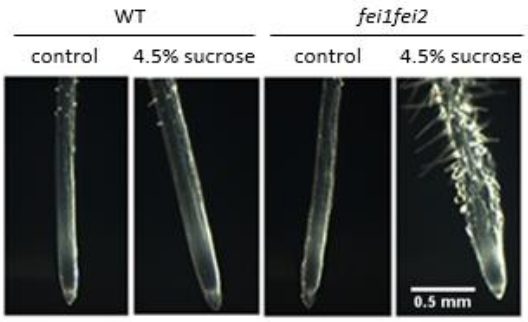

Figure 2: Root phenotypes of WT and *fei1 fei2* plants grown on control and high sucrose media (Johnson, unpublished)

Recent studies have indicated that ACC plays a role in regulating cellulose synthesis in *Arabidopsis*. Two highly similar Leucine Rich Repeat-Receptor Like Kinase (LRR-RLKs), named FEI1 and FEI2 have been identified as regulators of cell wall synthesis in *Arabidopsis* (Xu et. al. 2008). Mutations in the *FEI1* and *FEI2* genes disrupt the synthesis of cell wall, resulting in short, swollen roots when grown on high sucrose due to lower levels of cellulose (Fig. 2). Ethylene biosynthesis and perception was inhibited to determine the role of ethylene in the FEI pathway. Ethylene biosynthesis was blocked by treating mutant plants with aminooxyacetic acid (AOA), a compound that interferes with ACC, and α-amino[1-(14)C]isobutyric acid (AIB), a structural analog of ACC (Fig. 1B). Ethylene perception was inhibited through chemical means with 1-methylcyclopropene (1-MCP) and silver thiosulfate, or genetically using *ein2*, a strongly ethylene-insensitive mutant, or *etr1*, a null ethylene receptor mutant. Interestingly, blocking ethylene biosynthesis reversed the swelling in *fei1 fei2* mutants, but blocking ethylene perception did not (Fig. 3). Furthermore, a yeast two-hybrid assay showed that the kinase domains of both FEI1 and FEI2 interacted with ACS5 and ACS9, which are Type-2 ACS proteins (Xu et. al. 2008). This suggests that ACC might act as a signaling molecule, independent of ethylene, in the FEI pathway.

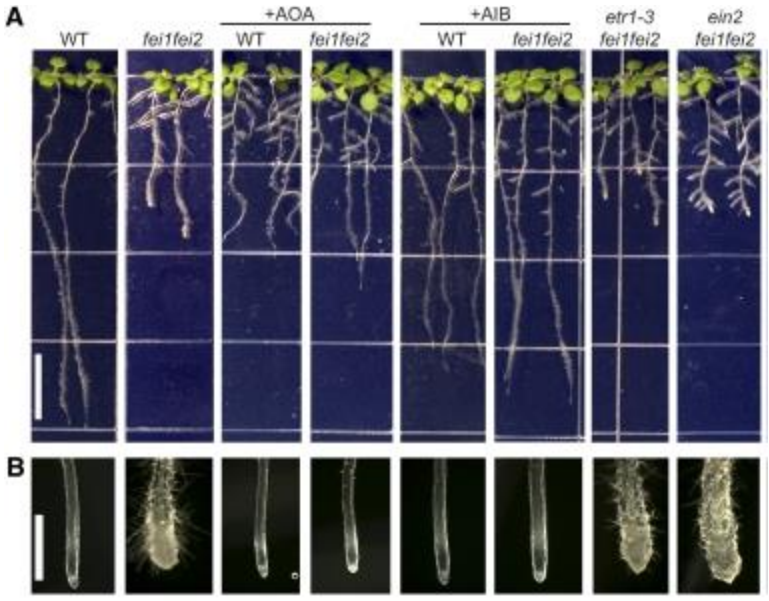

Figure 3: Root phenotypes of WT and *fei1 fei2* plants grown on high sucrose treated with nothing, AOA, or AIB. Treatment with AOA and AIB reverts the *fei1 fei2* mutant to WT phenotype, while blocking ethylene perception through genetic means does not (Xu et. al. 2008).

Several additional lines of evidence suggest that ACC is acting as a signal independent of its conversion to ethylene. First, root swelling caused by isoxaben, an inhibitor of cellulose biosynthesis, can be reversed by inhibition of ethylene biosynthesis, but not by inhibition of ethylene signaling (Tsang et al. 2011). Second, a higher order *acs* mutant (*acs2, 4, 5, 6, 7, 9, ami8, 11*) displayed embryonic lethality (Tsuchisaka et. al. 2009), but ethylene insensitive (*ein2*) mutants are viable (Guzzman and Ecker 1990). This suggests that ACC biosynthesis is essential for *Arabidopsis* viability, and that there likely exists an ACC signaling pathway independent of ethylene biosynthesis.

We are interested in determining the role of ACC in the FEI pathway in order to examine its role as an independent signaling molecule regulating cellulose biosynthesis. To determine if genetically reducing ACC levels results in a reversion of the *fei1fei2* phenotype, we are utilizing the CRISPR-Cas9 system to target *ACS* genes. We hypothesize that a null type-2 *acs* mutant will restore a wild-type phenotype in the roots of *fei1fei2* mutants. Furthermore, an octuple *acs* null

mutant (*acs2, 4, 5, 6, 7, 8, 9, 11*), obtained from crosses between type-2 and type-1 and -3 mutant lines, will be used to confirm the embryonic effects of *acs* null mutations (Tsuchisaka et. al. 2009). The CRISPR-Cas9 system will also be used to create a quintuple *aco* null mutant (*aco1, 2, 3, 4, 5*) to determine the effects of ACC buildup due to inhibition of ACC conversion to ethylene. We hypothesize that this buildup of ACC may enhance the *fei1 fei2* phenotype, leading to shorter, more swollen roots. Through these results, we seek to clarify the role of ACC as an independent signaling molecule in cellulose biosynthesis and plant development.

Results

CRISPR Constructs

The CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) system plays a role in bacterial adaptive immunity and has been adapted for gene editing in several organisms, including both monocot and dicot species of plants (Sanders and Joung 2014, Jiang et al. 2013). A guide RNA (gRNA) is used to target a specific region of a gene of interest. When the gRNA recognizes a complementary sequence in the gene, it recruits Cas9, an RNA-guided DNA endonuclease, to cause a double-stranded break in the target DNA (Li et al. 2013). When repair of the DNA takes place through non-homologous end joining (NHEJ), small insertions or deletions (INDELs) can occur in the coding region, thus causing mutation of the gene (Jiang et al. 2013).

CRISPR constructs were previously designed to target Type 1, 2, and 3 *ACS* genes in wild-type and *fei1 fei2* backgrounds. Two constructs, *ACS4-1,5-1,8-1,9-1,11-1* (named LR20) and *ACS4-2,5-2,8-2,9-2,11-2* (named LR21) were designed to target Type-2 *ACS* genes, and two constructs, *ACS2-1,6-1,7-1* (named LR23) and *ACS2-2,6-2,7-2* (named LR22) were designed to

target Type-1 and -3 *ACS* genes. A CRISPR construct to target *ACO1, 2, 3, 4, 5* genes (named LR24) was also designed. Wild-type and *fei1 fei2* plants were transformed with vectors containing CRISPR guides to give rise to T_1 seeds, which were then propagated to give rise to T_1 plants. The CRISPR vector also contained a BASTA-resistant gene that enabled the selection of transformed *Arabidopsis* plants. T_1 seeds were plated on media containing BASTA, and plants that displayed resistance were selected and allowed to self-fertilize.

Transformation with LR20 vector gave rise to 15 wild-type and 7 *fei1 fei2* T_1 lines while transformation with LR21 vector gave rise to 16 wild-type and 6 *fei1 fei2* T_1 lines; transformation with LR23 vector yielded 2 wild-type and 3 *fei1 fei2* lines while transformation with LR22 vector yielded 6 wild-type and 7 *fei1 fei2* lines. Lastly, transformation with LR24 gave 3 wild-type and 7 *fei1 fei2* lines. First generation CRISPR/Cas9 transgenic *Arabidopsis* plants contained mostly somatic mutations, but heritable mutations were found in subsequent generations, allowing for transmission of gene modification through generations.

Testing for CRISPR-Cas9-induced ACS and ACO gene editing using dCAPS Primers

Previously, we have used Polyacrylamide Gel Electrophoresis (PAGE) detect mutations that arose from CRISPR-induced editing events. Primers targeting a 60-base pair region around the CRISPR target site were used to create an amplicon that was analyzed for changes in length due to insertions or deletions (INDELs). While PAGE has produced reliable results in genotyping CRISPR mutations in rice (Burr, personal communication), INDELs in *Arabidopsis* are usually one base pair in length, which is too small for robust detection by PAGE. Thus, I have changed my INDEL analysis to an alternative method: CAPS (Cleaved Amplified Polymorphic Sequences) and dCAPS.

dCAPS (Derived Cleaved Amplified Polymorphic Sequences) technique is a modification of the CAPS technique (Neff et. al. 1998). The CAPS method utilizes differences in an existing restriction enzyme site to detect single nucleotide polymorphisms (SNPs). dCAPS primers are mismatched to the DNA template and create a restriction enzyme site in the DNA, which is then used to conduct restriction enzyme analysis to detect SNPs or, as used in my experiments, to detect INDELs (Hodgens et. al. 2017). CRISPR editing events can create insertions or deletions in the DNA sequence, causing a disruption in the restriction enzyme sites. If CRISPR-induced editing has taken place, the restriction enzyme fails to cleave DNA at that site (Fig. 4). In my experiments, dCAPS method have been used to detect expected editing sites with unknown INDEL events. The CRISPR editing region, identified by a Protospacer Adjacent Motif (PAM), was examined for existing or potential enzyme sites, and the appropriate CAPS/dCAPS primers were designed.

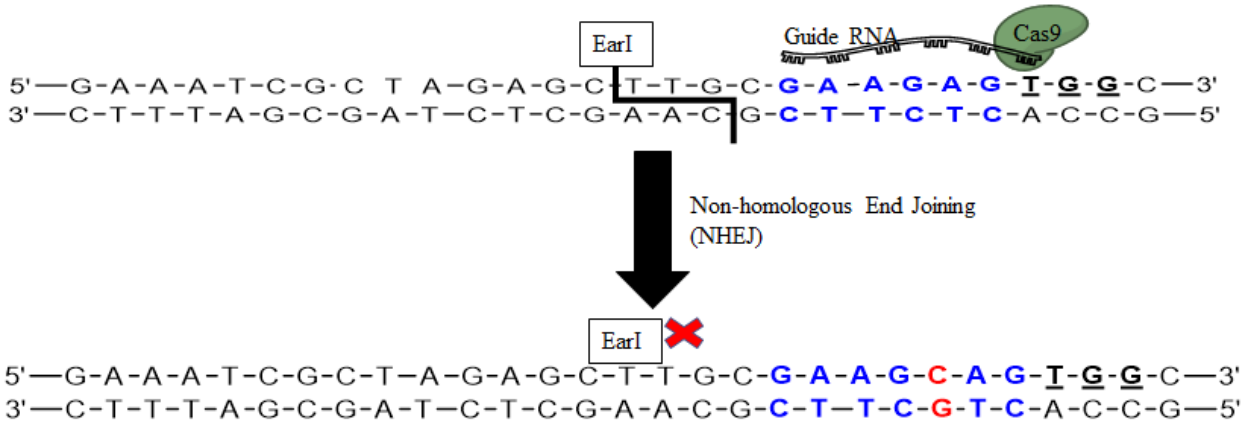

Figure 4: A cartoon showing editing event due to CRISPR-Cas9 cleavage and non-homologous end joining (NHEJ), and its detection through restriction enzyme analysis. The bases highlighted in blue represent the recognition sequence for the restriction enzyme, and the bases represented in red show an insertion event as a result of NHEJ. The length of the bands on agarose gel is used to determine whether editing has taken place in the gene. Unedited sequences will be cut by the restriction enzyme, will therefore be shorter and will travel farther on an agarose gel. Edited sequences will not be cut by the restriction enzyme and will therefore be longer and travel a shorter distance on the gel.

CRISPR-induced editing in ACS genes

The T_3 generation of plants transformed with the LR21 and LR23 vectors, in wild-type and *fei1 fei2* backgrounds were crossed, and the F_1 progeny was grown (Table S3). In total, 69 plants were genotyped for editing in the eight *ACS* genes using the appropriate CAPS/dCAPS primers and restriction enzymes on agarose gel (Fig. 5).

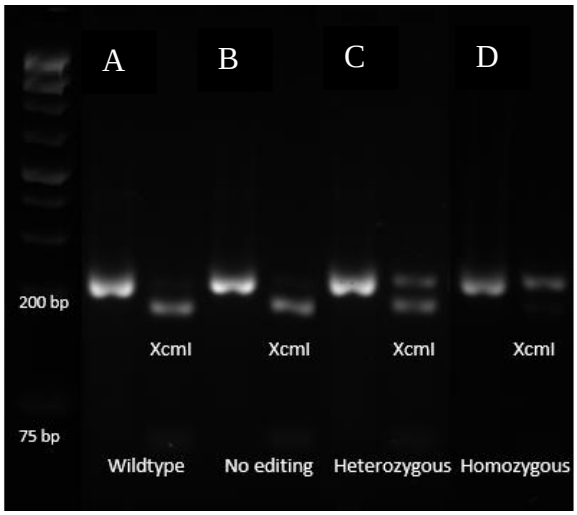

Figure 5: Example of agarose gel used to screen for mutations in *ACS2* gene. The first lane shows the undigested PCR product, and the second lane shows the PCR product digested with the XcmI restriction enzyme.

CRISPR targeting the *ACS2* gene was the most successful in editing in the wild-type background, with 90% of genotyped plants showing signs of editing (Fig. 6). It was also seen that 83% of *fei1 fei2* background plants showed signs of editing in *ACS2*. CRISPR targeting the *ACS4* gene was the most successful in editing in the *fei1 fei2* background, with 87% of genotyped plants displaying signs of editing. *ACS4* also had editing events in 72% of genotyped plants in the wild-type background. 64% of plants in the wild-type background and 70% of plants in the *fei1 fei2* background showed editing in the *ACS6* gene; 23% of plants in the wild-type background and 37% of plants in the *fei1 fei2* background showed editing in the *ACS9* gene; 13% of plants in the wild-type background and 27% of plants in the *fei1 fei2* background showed

editing in the *ACS11* gene. Editing was least effective in *ACS5, ACS7,* and *ACS8,* with none of the genotyped plants showing signs of editing in either the wild-type or the *fei1 fei2* backgrounds (Fig. 6).

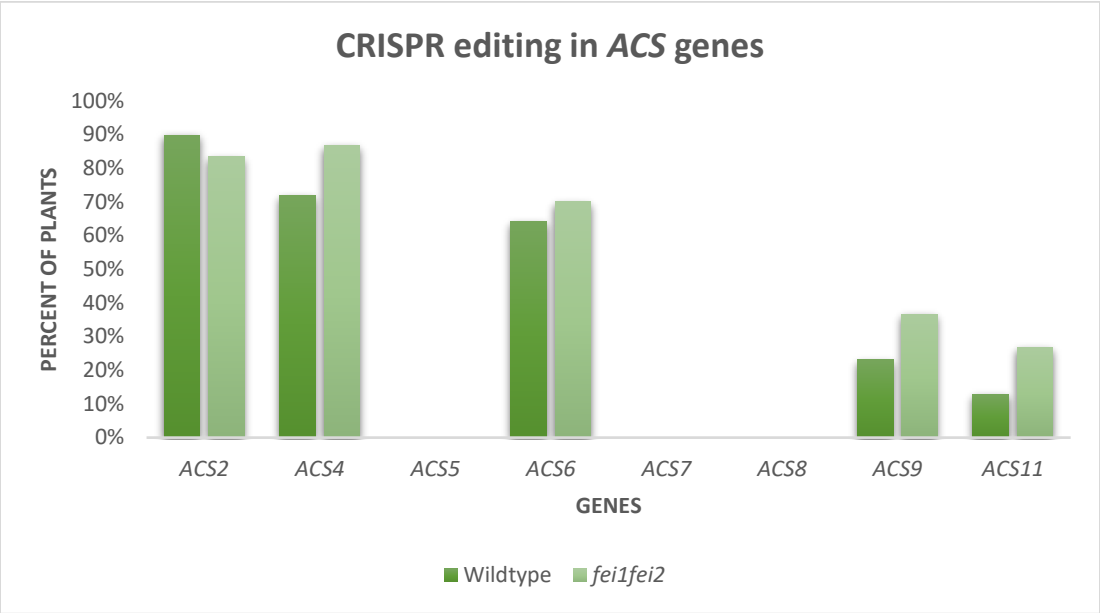

Figure 6: Percent of plants showing editing in *ACS* genes. The F_1 generation of crosses between plants carrying CRISPR guides for Type-2 and Type-1 and -3 *ACS* genes was tested.

Plants with higher order mutations, i.e. mutations in the most number of *ACS* genes, were chosen and the F_2 progeny was grown. In addition, plant lines that had been previously genotyped and shown to have CRISPR-induced mutations in *ACS5* and *ACS8* were also propagated. The propagated plants were screened for mutations in *ACS* genes, and then crossed to produce higher-order mutants. The seeds from these crosses were then propagated to give rise to the F_2 generation. These plants have since been subject to heat-shock treatment to increase efficiency of CRISPR-induced mutagenesis (LeBlanc et. al. 2017). They will be screened for mutations in the *ACS* genes (data not shown).

CRISPR-induced editing in ACO genes

T_2 wild-type and *fei1 fei2* plants carrying CRISPR guides targeting five *ACO* genes were selected and grown, and tested for CRISPR-induced editing in *ACO1, 2, 3, 4,* and *5* genes. In total, 61 plants were genotyped for editing using the appropriate CAPS/dCAPS primers and restriction enzymes on agarose gel (Fig 7).

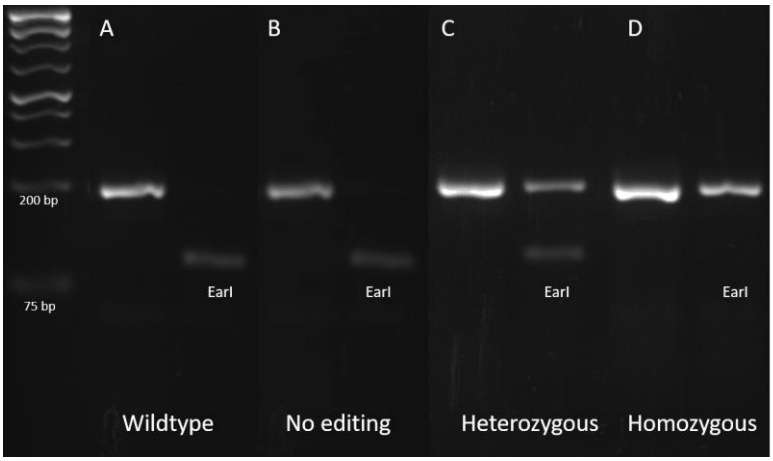

Figure 7: Example of agarose gels to screen for mutations in *ACO5* gene. The first lane shows the undigested PCR product and the second lane shows the PCR product digested with the corresponding restriction enzyme.

CRISPR targeting the *ACO1* gene was the most successful, with 97% of plants in the wild-type background and 60% of plants in the *fei1 fei2* background displaying signs of editing. 69% of plants in the wild-type background and 48% of plants in the *fei1 fei2* background showed editing in the *ACO2* gene; 6% of plants in the wild-type background and 20% of plants in the *fei1 fei2* background showed editing in the *ACO3* gene. Editing was least successful in the *ACO4* gene, with only 6% of plants in the wild-type background and no plants in the *fei1 fei2* background showing signs of editing. In the *ACO5* gene, 56% of plants in the wild-type background and 8% of plants in the *fei1 fei2* background showed editing (Fig. 8). Most plants that showed signs of editing were heterozygous for the edited gene. However, in LR24-9 plants,

four plants showed homozygosity for mutation in the *ACO5* gene (Fig. 7D), although the two alleles are edited differently and may not carry the same type of mutation.

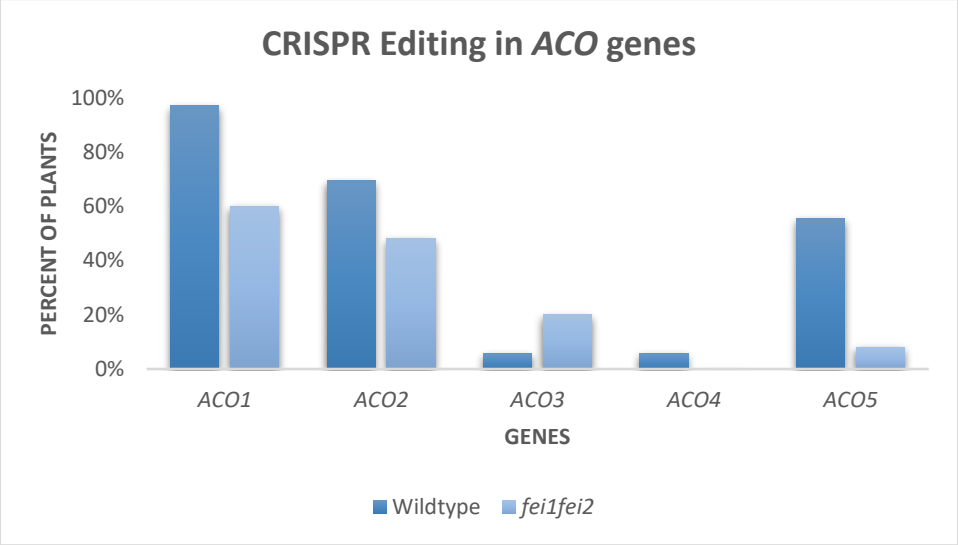

Figure 8: Percent of plants in the T_2 generation showing editing in *ACO* genes.

Plant lines with higher-order mutations, i.e. with editing in multiple *ACO* genes, were selected and propagated to the T_3 generation. These progenies were subjected to heat shock treatment to increase efficiency of CRISPR-induced mutagenesis (LeBlanc et. al. 2017). Preliminary screening of these plants for mutations indicates that the heat shock treatment has been successful in increasing the number of CRISPR-induced mutations (data not shown).

Discussion

Previous studies have indicated that ACC may act as a signaling molecule in the FEI pathway, which regulates cell wall synthesis in *Arabidopsis* (Xu et. al. 2008). The goal of this project is to create mutant lines that affect endogenous ACC levels in *Arabidopsis*, in order to study the role of ACC as a signaling molecule independent of ethylene biosynthesis. ACS proteins, which convert AdoMet into ACC, are encoded by eight genes, and ACO proteins,

which oxidize ACC to form ethylene, are encoded by five genes. CRISPR constructs were designed to target Type-2 *ACS* genes, Type-1 and -3 *ACS* genes, and *ACO* genes in order to create octuple *acs* and quintuple *aco* null mutant lines.

In *acs* mutant lines, preliminary results have shown that the CRISPR design for *ACS2* was most successful in the wild-type background and the CRISPR design for *ACS4* was most successful in the *fei1 fei2* background. Editing was not observed in *ACS5, ACS7,* and *ACS8* in the plant lines that were genotyped, but has been observed previously in other plant lines. In the *aco* mutant lines, preliminary results have shown that CRISPR design for the *ACO1* gene was the most successful in editing, while the CRISPR design for *ACO4* was the least successful in editing. Heat-shock treatment, which has been shown to increase efficiency of mutagenesis by CRISPR-Cas9, was administered on recently grown plant lines. Exposing the plant to heat stress at 37°C allows for Cas9 to create more double-stranded breaks than at 22°C, thus increasing the mutation rate of CRISPR-Cas9 in *Arabidopsis* (LeBlanc et. al. 2017). Mutant lines exposed to heat stress will be screened, and higher-order mutants will be chosen in both the wild-type and *fei1 fei2* backgrounds.

In individual plants, the region surrounding the PAM site will be amplified and sequenced in order to see what type and precisely what size of INDEL event has taken place. In *Arabidopsis*, double-stranded break repair most commonly results in single base pair INDEL events, although they can be up to five base pairs in length (Feng et. al. 2014). Frameshift mutations, resulting from insertions or deletions of 1, 2, 4, or 5 base pairs, are most desirable. A 3 base pair change, which results in the addition or deletion of one amino acid in the polypeptide chain, may not affect the structure of the protein severely and thus, may not lead to a null mutation. We will therefore not choose plants with 3 base pair changes.

Plants will then be screened for segregation of the Cas9 vector in order to remove the Cas9 gene from the plant while keeping CRISPR-induced mutations. Plants heterozygous for Cas9, when self-fertilized, will yield a Mendelian ratio of 3:1, where 75% of the progeny will contain Cas9 and 25% will not. We seek to remove Cas9 from the system in order to prevent off-target mutations from occurring. An off-target mutation may lead to phenotypic effects unrelated to the studied mutations, which would complicate the analysis of the study.

Higher-order mutant lines will be propagated and crossed in both the wild-type and *fei1 fei2* backgrounds, until octuple *acs* null mutant lines and quintuple *aco* null mutant lines can be obtained. It is hypothesized that Type-2 *acs* mutants, containing mutations in *ACS4, 5, 8, 9,* and *11* genes, will reverse the *fei1 fei2* root swelling phenotype to a wild-type morphology. Since ACS activity is inhibited, AdoMet will not be converted to ACC, leading to reduced ACC levels. It is hypothesized that this might have similar effects as treating the plant with AOA or AIB and should rescue the *fei1 fei2* mutant phenotype. The octuple *acs* null mutant will also be characterized for effect on the *fei1 fei2* phenotype. Additionally, the octuple mutant will be tested for phenotypes such as lethality to confirm previous findings. The quintuple *aco* mutant will inhibit ACO activity within the plant, which will prevent the plant from converting ACC to ethylene and will lead to elevated levels of ACC. It is hypothesized that this increase in ACC will enhance the *fei1 fei2* mutant phenotype, either leading to shorter, more swollen roots, or by the occurrence of the *fei1 fei2* root swelling phenotype when grown on media containing low levels of sucrose. These results will illuminate the role of ACC as a signaling molecule in the FEI pathway, and shed light on its effects on cell wall synthesis in *Arabidopsis thaliana*.

Materials and Methods

Plant Growth

To sterilize seeds, they were washed in 95% ethanol and then incubated in a bleach solution (20% bleach, 50% tween-20 [Fischer Scientific]) for 5-10 minutes. Seeds were then washed with deionized water until the smell of bleach no longer persisted. To select for plants with the CRISPR/Cas9 vector, seeds were grown on MS media containing phytagel (Sigma Lifescience) at 6g/L, MS-MES (Research Products International) at 4.8g/L, and BASTA® (Gold Biotechnology) at 50μg/mL. Seeds were transferred onto the prepared media using pipette tips. They were then left at 4°C for four days, and then grown at 22°C until the resistant seedlings could be differentiated. The seedlings were then transferred to soil and grown in 24-hour light.

Designing and Testing CAPS/dCAPS Primers

The region around the PAM site, where CRISPR/Cas9 targets, was manually analyzed for existing and potential restriction enzyme sites using the SnapGene Viewer software. The designed primers are given in Table S1 and S2. CAPS and dCAPS primers were used to flank CRISPR target sites in order to amplify ~200 base pairs around the target region. CAPS primers do not affect restriction enzyme sites, whereas dCAPS primers introduce restriction enzyme sites by using a DNA strand mismatched to the DNA template. Wild-type DNA was used to run a temperature gradient PCR between 45°C and 65°C in order to determine the optimal annealing temperature for each forward and reverse primer pair. Restriction enzyme analysis was also performed using the designed primer pairs and wild-type *Arabidopsis* DNA to test that the dCAPS primers successfully introduced a restriction enzyme site.

DNA Extraction

To extract DNA from plants, a leaf from each plant was first taken and placed into a DNA extraction tube with 2 metal beads. The DNA extraction box was then cooled using liquid nitrogen and run in the Geno/Grinder® (SPEX SamplePrep). The ground samples were incubated with CTAB extraction buffer at 65°C for approximately 30 minutes. Chloroform (Macron Fine Chemicals) was added, and the samples were centrifuged at 15000 rotations per minute (rpm) for 5 minutes. The aqueous layer of the resulting solution was transferred to another tube. Sodium acetate and isopropanol (Macron Fine Chemicals) were added to precipitate DNA. The solution was centrifuged at 15000 rpm for 5 minutes, and the liquid was cleared using the Aspirator Pump (Cole-Parmer Instrument Company). 700µL of 70% ethanol was used to wash the DNA pellet. The solution was centrifuged and the liquid was cleared using the Aspirator Pump. The pellet remaining in the tube was then dried for 30 minutes, and then suspended in water. The DNA was then stored in -20°C.

Genotyping for CRISPR Editing

To check for CRISPR editing, a 200- base pair region around the PAM site was amplified using PCR at standard conditions. The PCR product was then digested using the appropriate restriction enzyme and buffer (NEB) at conditions specified for the restriction enzymes, as shown in Table S3. A restriction analysis was then run using gel electrophoresis. The PCR product was run on 3% agarose (Fischer Scientific) gel containing 1X TBE buffer and 2.5µL/mL ethidium bromide (Fischer Scientific) at 130V for approximately 30 minutes. The gel was then imaged using ChemiDoc Touch Imaging System (Bio-Rad).

Heat Stress Treatment

Plants, once transferred to soil, were allowed to acclimate to soil conditions for one week, until growth of new leaves was detected. The plants were transferred to 37°C for 30 hours, and then allowed to recover at 22°C for 42 hours. The plants underwent four such treatment cycles.

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Supplemental Tables and Figures

Table S1: Primers and restriction enzymes used to investigate the gene for editing in *ACS* gene. Red letters in the primer sequence indicate a mismatch in the dCAPS primer to the template DNA, where the highlighted base has been added to the primer.

Gene	Name	Primer Name	Primer Sequence	Enzyme
ACS4	*acs4-1*	dCAPS_ACS4-1F_BslI	TACATGCAACAGCCATGGCCAAGTCTCTTCGTATTTACTT	BslI
		dCAPS_ACS4-1R_BslI	TGGATAATGCCTTGAGGGTTCTTGGTACCGT	
	acs4-2	392 crACS4-2F	CAGAGAGACTAATTTAAAGT	HinfI
		CAPs_ACS4-2Rev	GTTCCCGGAAAACAGACTGG	
ACS5	*acs5-1*	dCAPS_ACS5-1Fwd	AGAAGAATCCTTATGATGAGATCCAGAACC	XcmI
		dCAPS_ACS5-1Rev	TTGAATTCAGGCATGCCATG	
	acs5-2	dCAPS_ACS5-2Fwd	GACAAGCAATGGTCATGGAC	BslI
		dCAPs_ACS5-2Rev	CATGACTCGATTAGATCGAAACATAGCCGG	
ACS8	*acs8-1*	dCAPS_ACS8-1Fwd	ACGACGAGATCAAGAACCCAGACGCCATTA	XcmI
		crACS8-2R_60bp	CGAAATATGGATTGGCCTTC	
	acs8-2	dCAPS_ACS8-2Fwd	TTGAGTCATGGCTTGCTAAGAACGCCGAC	MwoI
		dCAPS_ACS8-2Rev	GGTTACGTTGTCATATCGTT	
ACS9	*acs9-1*	dCAPS_ACS9-1Fwd	CGACGAAATCAAGAACCCTAATGCCATTAT	XcmI
		crACS9-2R_60bp	GAAAATGGATTGGCCGTCCT	
	acs9-2	dCAPS_ACS9-2Fwd	TTAGCTAAGAATCCGGACGCAGCCGGCCTA	BslI
		ACS9seqR	CTTGCTTGGATCAAATGTTA	
ACS11	*acs11-1*	dCAPS_ACS11-1Fwd	TTCTTTTTGCAGCTTTCTTTTGACCCAATA	XcmI
		dCAPS_ACS11-1Rev	CTTACATCCTTGAAAGCTGGCAAGCAATGG	
	acs11-2	406 crACS11-1F	GTACACAATTTCCAAACTTT	BslI
		dCAPS_ACS11-2Rev	ATATGTTACTTACATCCTTGAAAGCTGCCAA	
ACS2	*acs2-1*	dCAPS_ACS2-1Fwd	CATCTTTCCCGTAACCCCCATGCCATCATC	XcmI
		dCAPS_ACS2-1Rev	TAGCTTGATGTGTATACGTG	
	acs2-2	CAPS_ACS2-2Fwd	GAACCCAGAAGCTTCTATTT	XcmI
		413 crACS2-2R	GTAGCTGATTACAAGATATC	
ACS6	*acs6-1*	dCAPS_ACS6-2_AleI	GATCTGAATCTATTGTCTAAAATCCACTCC	AleI
		CAPs_ACS6-1Rev	TGATTTTCAGCGAGACCCAT	
	acs6-2	dCAPS_ACS6-2Fwd	GAAGAAAACCCATTTCACCC	BsaWI
		dCAPS_ACS6-2Rev	ATCGAAGCTTCTGGATGTTTTAAAAGCCAT	
ACS7	*acs7-1*	dCAPS_ACS7-1_XcmI	GAAACTTACTTGGAGAAGAAGAATCCACCA	XcmI
		ACS7seqRev	AGTTCGTTAGCGGCGGTGGC	
	acs7-2	418 crACS7-1F	CAAACAGGTCTCGTTTGATC	BslI
		dCAPS_ACS7-2Rev	GGTAGTCTTGAAACAATGCGTTTTCACCGA	

Table S2: Primer and restriction enzymes used to investigate gene editing in *ACO* genes. Red letters in the primer sequence indicate a mismatch in the dCAPS primer to the template DNA, where the highlighted base has been added to the primer.

Target Site	Primer Name	Primer Sequence	Enzyme
ACO1 (AT2G19590)	ACO1fwd_caps_2	ATGTCACTTCTTGATCATGCA	MboII
	ACO1rev_caps_2	TCTTCTTCACTTTCTCCATCA	
ACO2 (AT1G62380)	ACO2fwd_CAPs	TTACTTTCAAAGAAGAGAGAGA	BslI
	ACO2rev_CAPs	AGCTATAGTCAAATCTTTTCAAACA	
ACO3 (AT1G12010)	ACO3fwd_dCAPs	AAACCATGGCTTTGATCGACGATCCATGT	XcmI
	ACO3rev_dCAPs	CTGAAATAACGAAACTTTGAATTGT	
ACO4 (AT1G05010)	ACO4fwd_dCAPs	TATGGAGAAGATCAAAGACCCATGT	XcmI
	ACO4rev_dCAPs	TCTCTTCCATGCACTTCTTGTAA	
ACO5 (AT1G77330)	ACO5fwd_CAPs	TCTCTCTTTGCGGATCTGAAATGG	EarI
	ACO5rev_CAPs	CATACACAGATAAGCACATGAA	

Table S3: Restriction enzymes used in the CAPS/dCAPS genotyping and restriction conditions.

Enzyme	Buffer	Incubation Temp (°C)	Incubation Time (hr)	Heat Inactivation Temp(°C)	Heat Inactivation Time (min)
XcmI	NEB2.1	37	4	65	20
AleI	Cutsmart	37	2	80	20
MwoI	Cutsmart	60	2	no	no
BslI	Cutsmart	55	2	no	no
HinfI	Cutsmart	37	2	80	20
BsaWI	Cutsmart	60	2	80	20
MboII	Cutsmart	37	2	65	20
EarI	Cutsmart	37	2	65	20

Table S4: Crosses set up between T_3 plants containing CRISPR vectors targeting *ACS* genes, and the number of plants propagated from each of these crosses.

Crosses	Background	Number of propagated plants
LR21-9-21 x LR23-6-17	Wild-type	11
LR23-6-17 x LR21-9-21	Wild-type	11
LR23-6-21 x LR21-9-23	Wild-type	4
LR21-9-23 x LR23-6-21	Wild-type	13
LR23-37-24 x LR21-44-24	*fei1 fei2*	9
LR23-37-20 x LR21-44-21	*fei1 fei2*	4
LR21-44-21 x LR23-37-20	*fei1 fei2*	6
LR21-44-24 x LR23-37-24	*fei1 fei2*	11