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Evaluating Potential Plant Hormone Cross Talk between Auxin and Ethylene in Arabidopsis

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EVALUATING POTENTIAL PLANT HORMONE CROSS TALK BETWEEN AUXIN AND ETHYLENE IN *ARABIDOPSIS*

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DEDICATION

I dedicate my thesis work to my family and friends. A special feeling of gratitude to my loving parents, Marcel and Elizabeth Brown, whose words of encouragement and fervent prayers pushed me to complete the work started within me. To my sister Hope, who has never left my side and is very special and dear to my heart.

I also dedicate this thesis to my many friends who have supported me throughout the process. I will always appreciate all they have done, especially Janae Fields for constantly reminding me of the greater call on my life, Tiffany Rhodes for helping me stay upbeat when times got tough, and Eugene Lacey for being a great supporter in our college and post-graduate science careers.

I dedicate this work and give special thanks to my mentor Dr. Marcia Harrison. She is a woman with many titles, but the one most cherished one to me is “friend”. Over the past twelve years I have seen her consistently make it a focus to empower women, help others succeed, and give recognition and redirection to aid in the process of developing others, such as myself. Words cannot express the honor and privilege it was to work with such an extraordinary woman who far exceeds what it is to be a leader, mentor, and a friend.

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LIST OF ABBREVIATIONS

ABRC: The Arabidopsis Biological Resource Center

ACC: 1-aminocyclopropane-1 carboxylic acid

ACO: 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE protein

ACS: 1-AMINOCYCLOPROPRANE-1 CARBOXYLIC ACID SYNTHASE protein

ACS5p::GUS: ACS5promoter::GUS

AdoMet: 1-adenosylmethionine

AGRIS: Arabidopsis Gene Regulatory Information Server

ARF: AUXIN RESPONSE FACTOR protein

AuxRE: auxin response element

AUX/IAA: AUXIN RESISTANT protein

CTR: CONSTANT TRIPLE RESPONSE protein

EIN: ETHYLENE INSENSITIVE protein

ERE: ethylene response element

ERF: ETHYLENE RESPONSE FACTOR protein

GCC: Guanine-Cytosine-Cytosine motif

GUS: β-glucuronidase

IAA: indole-3 acetic acid

PERE: primary ethylene response element

TAIR: The Arabidopsis Information Resource

T-DNA: Transfer-DNA of the plasmid of *Agrobacterium tumefaciens*

TIR: TRANSPORT INHIBITORY RECEPTOR protein

WT: Wild type

ABSTRACT

Auxin is the primary hormone responsible for plant growth and development. Regulation of auxin-response genes occurs through transcriptional activators (auxin response factors called ARFs) which bind to auxin response elements (AuxREs). Some auxin-responsive genes encode aminocyclopropane-1-carboxylic acid synthase (ACS) enzymes which regulate the production of the plant hormone ethylene. The major research objective was to evaluate transcriptional cross talk between auxin and ethylene. Both AuxREs and ethylene response elements were found in several *ACS* and *ARF* genes, suggesting cross talk between the two hormones at the transcriptional level. Analysis of transgenic *Arabidopsis thaliana* plants deficient in *ARF7* and containing a reporter gene for the *ACS5* expression were used to evaluate cross talk supports ARF7 regulation of *ACS5* expression after auxin treatment in dark-grown seedlings and during flower development in light-grown seedlings, but do not show a strong association with gravitropism and wounding responses.

CHAPTER 1.

INTRODUCTION

Plant organs grow and function because of hormones within the plant. Hormones made in one cell or tissue will communicate to another cell or tissue through a common communication vehicle, known as signal transduction, to modulate growth and development. Within six major types of plant hormones that can potentially modulate signal transduction, two hormones, in particular, play a special role of interest. The first is the primary plant growth hormone auxin, which stimulates cell elongation and occurs as indole-3-acetic acid (IAA) in nature. The second is the gaseous hormone ethylene which generally has an inhibitory role in growth. Elevated ethylene levels inhibit cell elongation, induce leaf abscission, and may stimulate cell death. Also, ethylene production is induced by auxin treatment and wounding.

Arabidopsis thaliana, The Plant Genetic Model

Hormonal responses of auxin and ethylene have been modeled in a small flowering plant from the mustard family (*Brassicaceae*), *Arabidopsis thaliana*. *A. thaliana* is the plant genetic model used for basic research in plant genetics and cellular and molecular biology of flowering plants. Although the plant has no significance to agriculture, it is advantageous for research because of its short life cycle and its ability to produce a large amount of seeds even when grown in a restricted area. *A. thaliana* was the first plant to have its genome sequenced, and approximately 90% of its genome has been sequenced and annotated. Genetic and physical maps of its five

chromosomes, along with other information about this organism are made public and are available through The Arabidopsis Information Resource (TAIR) webpage.

The highly detailed gene information and numerous tools available for *A. thaliana*, make it central for studying auxin and ethylene interactions for this research project. One goal of the *Arabidopsis* genome project was to develop knock-out or loss-of-function genes for all *A. thaliana* genes. *A. thaliana* is usually referred to as *Arabidopsis* in the literature and follows the conventions for gene nomenclature for plants. Therefore, wild type gene names are abbreviated by three-letter initials which are capitalized and italicized. For example the *AUXIN RESPONSE FACTOR* gene is abbreviated as *ARF*. Mutant forms of genes are indicated by the lower case, italicized initials. For example, the mutant form of *ARF* is *arf*. The protein product is indicated by capitalized and non-italicized initials, such as ARF. Variations of genes are indicated by different numbers following the abbreviation. For example, ARF7 is a specific type of auxin response factor protein. Different alleles of the same gene are indicated by a hyphen followed by a number. For example, *AXR1-12* indicates the gene for *AUXIN RESISTANT*, AXR1 and the 12 version of the gene (or allele). When there is only one allele known, then no hyphenated number is indicated, such as *ARF7*.

The TAIR webpage links to the annotated gene and protein information and is also a portal to many genetic tools for *Arabidopsis*. For this study, tools to identify and evaluate the DNA sequence of the promoter regions of the genes of interest were used.

Ethylene – Overview and Biosynthesis

The plant hormone ethylene is used by plants as a signaling molecule integral for regulating a variety of stress responses and developmental processes such as seed germination, fruit ripening, and cell elongation. Ethylene production is induced by a variety of external factors such as wounding, temperature changes, and drought conditions. Internally, ethylene is also induced by changes in auxin levels. Ethylene plays a key role in shoot development, controlling the elongation of the hypocotyl, the transition tissue that is between the seed leaves called cotyledons, and the root (Abeles et al., 1992). A characteristic effect of ethylene is its “triple response” which causes reduced elongation, increased thickness, and altered orientation of hypocotyls. This response was experimentally used as a bioassay for ethylene.

Ethylene production increases in response to wounding or increased auxin treatment. For example, when dark-grown pea plants were mechanically wounded, ethylene production increased after a lag of 26 minutes, to a maximum of nearly over four times within one hour after wounding (Saltveit and Dilley, 1978). This characteristic lag followed by a rapid increase is found in most plant tissues, but the timing and magnitude of the response varies between species and tissue types. When treated with auxin, ethylene production increases over time.

High levels of auxin have also been found to inhibit stem hypocotyl elongation. Experimental evidence indicates that this inhibition is caused by auxin-induced ethylene biosynthesis. Therefore, under conditions in which auxin accumulates in the stem, ethylene is produced, resulting in growth inhibition as a form of feedback regulation. For example, when 35-45-day-old *Arabidopsis* plants were treated with 100 μM IAA,

ethylene levels increased in the inflorescence (compound flower stems) stalks, root tips, and young leaves (Arteca and Arteca, 2008). However, when auxin resistant mutants *axr1-12, axr1-3,* and *axr2* were exposed to 100 μM IAA, these mutants produced lower amounts of ethylene compared to wild type (Arteca and Arteca, 2008) but to different extents. For example, *axr1-12* produced slightly less IAA-induced ethylene than the wild type; whereas auxin resistant mutant *axr1-3* produced lower levels of IAA-induced ethylene than *axr1-12,* while *axr2* barely produced detectable levels of ethylene (Arteca & Arteca, 2008). These findings demonstrated that reducing the auxin response also lowers ethylene, thus supporting a stimulatory role for auxin in ethylene production.

Ethylene biosynthesis begins with the amino acid methionine, which is modified to produce 1-adenosylmethionine (AdoMet) (Figure 1). In this pathway originally described by Yang and Hoffman, AdoMet converted to 1-aminocyclopropane-1-carboxylic acid (ACC), and ethylene is produced by the oxidation of this precursor (Yang and Hoffman, 1984). The production of ACC by ACC synthase (ACS) is considered the primary regulatory step in the ethylene biosynthetic pathway. For example, *Arabidopsis* seedling hypocotyl length showed inhibition similar to increased ethylene production when grown on plates containing ACC (Collett et al., 2000). It is assumed that the ethylene production is dependent on the level of ACC and the ACC oxidase enzyme is seldom saturated (Wang et al., 2002). Therefore, increased levels of ACC usually result in increased ethylene production. In general, research which evaluates regulation by the ACS enzyme is used to understand ethylene biosynthesis, especially for the regulation of plant development.

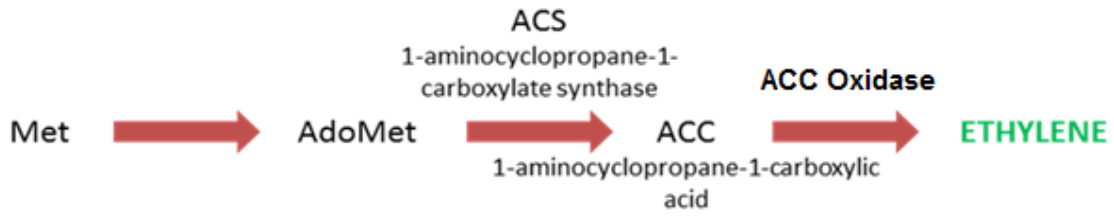


Figure 1. An overview of the ethylene biosynthetic pathway. The regulation of ACC synthesis by ACC synthase (ACS) serves as the primary rate-controlling step in ethylene biosynthesis, since ACC oxidase (ACO) converts ACC readily to ethylene in the presence of oxygen. This enzyme can be regulated by external factors such as auxin and wounding (Wang et al., 2002).

Studies of ACS revealed that it is a group of related enzymes that are encoded by a multigene family whose expression is differentially regulated in various tissues in every plant species examined (Wang et al., 2002). In *Arabidopsis* the *ACS* gene family encodes for 8 functional *ACS* forms (*ACS1*, *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS8*, *ACS9*, and *ACS11)*. Members of this multigene family show specific tissue expression under various controlled conditions. For example, in a wounding study, the hypocotyl tissue of five-day-old light-grown *Arabidopsis* seedlings were cut and the differential *ACS* expression was evaluated (Tsuchisaka and Theologis, 2004a). After wounding, *ACS1* and *ACS5* expression was seen throughout the hypocotyl tissue, *ACS*2, *ACS*4, *ACS*6, *ACS7*, *ACS8*, and *ACS11* were expressed in the basal part of the hypocotyl tissue, whereas *ACS9* showed no expression (Tsuchisaka and Theologis, 2004a). In a further study, exogenous treatment of auxin also showed specific tissue expression for the various *ACS* family members. Treatment with 20 μM IAA to the roots of five-day-old light grown *Arabidopsis* seedlings, enhanced the expression of *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, and *ACS11*, while the expression of *ACS1* and *ACS9* was not changed by IAA (Tsuchisaka and Theologis, 2004a).

Changes in *ACS* expression in response to wounding and auxin treatment are often attributed to transcriptional regulation. When *Arabidopsis* seedlings were exposed to auxin, various levels of *ACS* expression were observed in different cells throughout the root tip. These results suggested that auxin is differentially induced depending on the cell type and the specific *ACS* gene isoform (Tsuchisaka and Theologis, 2004a). Localization of expression may be due to the absence of repressor proteins. For example, *ACS* gene family members, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, A*CS9* and *ACS11* are regulated by auxin (Tsuchisaka and Theologis, 2004a) and can be suppressed by Aux/IAA transcription proteins (Figure 4) in the regulatory region of the *ACS* gene. Repressing *ACS* gene expression prevents the transcription of *ACS*, thus, inhibiting ethylene production, since production of ACC is generally considered the rate controlling step in ethylene biosynthesis. As mentioned previously, the auxin insensitive mutants *axr1-12, axr1-3,* and *axr2* produced lower levels of ethylene compared to that of wild type after auxin treatment when exposed to 100 μM IAA (Arteca and Arteca, 2008).

Ethylene signal transduction

Increased ethylene production triggers a signal transduction cascade which activates ethylene-responsive genes (Figure 2). When ethylene is present, it will bind to receptors located on the ER membrane (Van Zhong and Burns, 2003). These ethylene receptors act as negative regulators of ethylene responses, thus ethylene binding to these receptors inactivates them, which causes downstream suppression of other key negative regulators in this pathway (Figure 2) (Van Zhong and Burns, 2003). In the

absence of ethylene, CTR1 (CONSTANT TRIPLE RESPONSE 1), a negative protein regulator to ethylene responses, will suppress ethylene responses downstream. However, as a result of the binding of ethylene to its receptor, CTR1 will be inactivated and no longer suppresses ethylene responses. Thus, the ethylene responses are ultimately under the control of the resulting activation and interaction between an integral membrane protein that is a positive regulator to ethylene responses, EIN2 (*ETHYLENE INSENSITIVE2*), and a transcription factor, EIN3 (Figure 2). In the nucleus, EIN3 binds specifically to a primary ethylene response element (PERE) in the promoter region of its primary response gene, *ETHYLENE RESPONSE FACTOR1*, *ERF1*. EIN3 binding to the PERE will activate gene expression of *ERF1* (Figure 2). After *ERF1* is transcribed and translated, it will bind to a GCC-box present in the promoters of many ethylene-regulated genes (secondary genes). In addition, other ethylene response elements (EREs) are located in the promoter regions of ethylene-induced genes that are targets for EIN3 binding as well. Therefore, EIN3 can also regulate many other genes in response to ethylene (Figure 2).

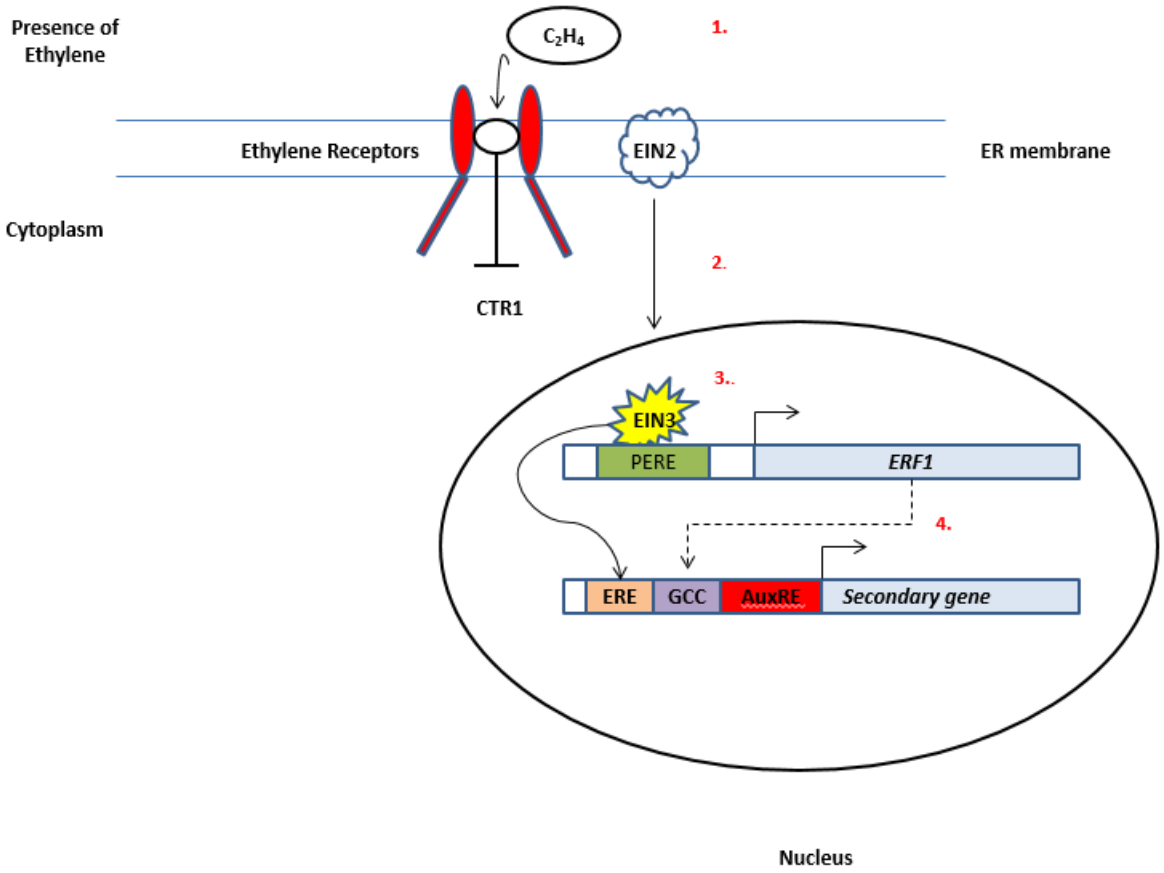


Figure 2. Model of ethylene signal transduction pathway. 1. Ethylene binding inactivates the ethylene receptor and CTR1. 2. Inactivation of CTR1 (indicated by a T-bar) allows for integral membrane protein EIN2 to positively regulate (indicated by a solid arrow) ethylene responses. 3. Activation of EIN3 by EIN2 regulates the gene expression of primary response gene *ERF1*. 4. Transcription and translation of ERF1 are represented by the dashed line. The ERF1 binds to a specific GCC box in the promoter region of secondary ethylene response genes. EIN3 can also bind to other EREs in the promoter region of secondary ethylene response genes to regulate gene expression (not shown).

Auxin

Auxin is the primary hormone responsible for plant growth and development. Some developmental processes regulated by auxin include embryo and fruit development, elongation growth, apical hook formation in seedlings, and root branch patterning (Kepinski and Leyser, 2005). In nature, auxin occurs as indole-3-acetic acid (IAA), while synthetic auxins generated by agricultural and horticultural industries include naphthalene acetic acid, indole butyric acid, 2, 4-dichlorophenoxyacetic acid,

and 2-methyl-4-chlorophenoxyacetic acid. Naphthalene acetic acid is an herbicide used to control tree growth after pruning. Indole butyric acid is used to increase root development and produce riper fruit. Very active synthetic auxins, 2, 4-dichlorophenoxyacetic acid and 2-methyl-4-chlorophenoxyacetic acid, are primarily used to inhibit the growth of broad leafed plants, specifically weeds acting as herbicides.

Auxin is primarily made in the shoot apical meristems and young leaves of a plant (Taiz and Zeiger, 2006). Within a plant, auxin distribution varies in different tissue types, but is maintained within all plant tissues. Higher levels of auxin are evident in young fruits, seeds, and in the shoot and root tips, where it is synthesized.

Auxin regulates gene expression through signal transduction pathways and can alter gene expression in specific areas of a tissue where it accumulates. For example, when auxin accumulates in the lower part of the stem of a horizontally-reoriented plant, it stimulates growth on the lower side of the stem causing the stem to bend upward. Changes in auxin distribution have been experimentally visualized by transforming plants with a *DR5::GUS* (β-glucuronidase) reporter gene construct that is expressed where there are higher levels of auxin in the tissue (Li et al., 1991). Under normal growing conditions in the soil, an *Arabidopsis* seedling is vertical in orientation and auxin is equally distributed (higher auxin is indicated by the blue color) (Figure 3A). This equal distribution results in straight growth. However, when a seedling is placed in a horizontally-oriented position, auxin is distributed asymmetrically (Figure 3B). In this case auxin concentration increases on the lower side stimulating that side to curve upward in a process called gravitropism. Because auxin can accumulate as a result of a plant's reorientation of its original gravitational field, it can differentially regulate gene

expression in the lower portion of the stem, specifically in the genes responsible for increased cellular elongation. Thus, as the cells in the lower part of the stem elongate, this drives the plant's response to gravity, and the plant's ability to reorient itself back to its original position.

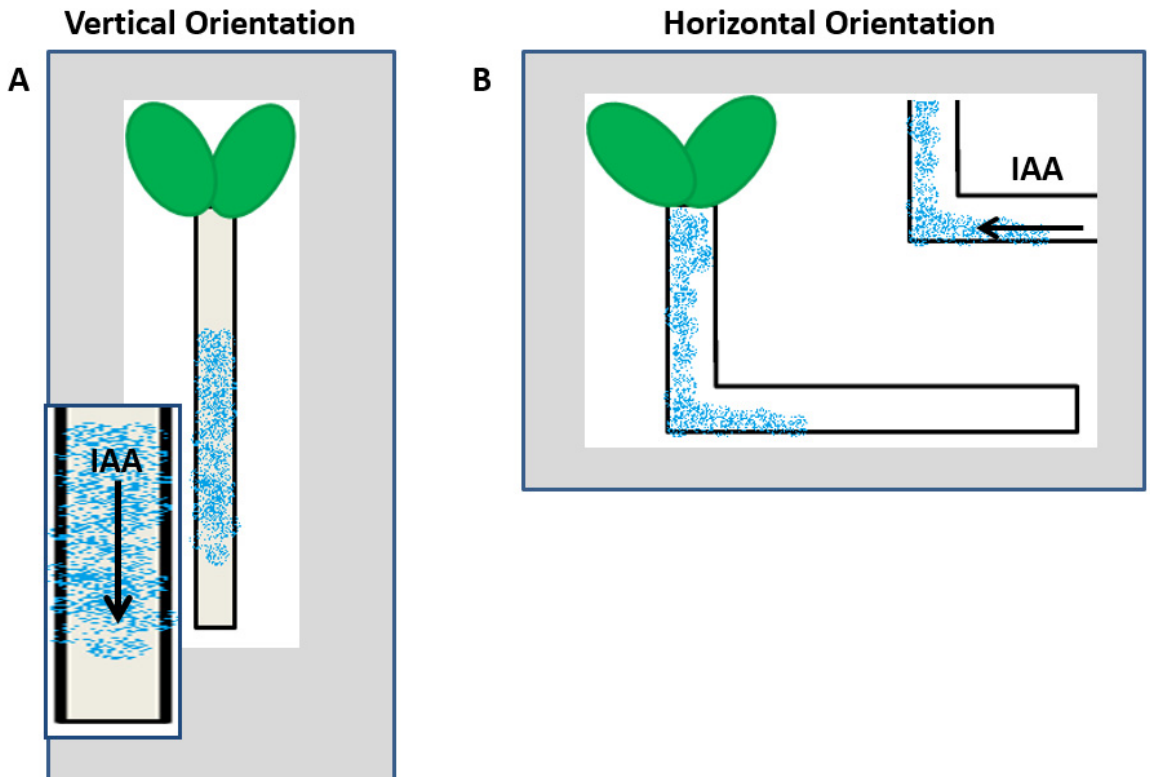


Figure 3. Cell elongation is the driving force of the plant's response to gravity. (A) In a vertically-oriented *Arabidopsis* seedling, auxin (indicated by blue) is equally distributed and results in straight growth development. (B) In a horizontally-oriented *Arabidopsis* seedling there is an asymmetric distribution of auxin that results in unequal growth and upward curvature.

Therefore, studies that evaluate gravitropism should also include the regulation of the ACS enzyme and the role of ethylene production in the plant. In fact, a study conducted by Burg and Clagett reported that the presence of auxin enables the conversion of methionine to ethylene (Burg and Clagett, 1967). When auxin reached higher concentrations, it stimulated ethylene production and inhibited cell elongation. In addition, ethylene has been observed to increase in horizontally-placed stems

(Harrison, 2007). This increase has been attributed to inhibiting auxin-induced growth, and slowing of the curvature as the plant reaches a vertical position, thus contributing to the complex kinetics of the gravitropic response.

The regulation of auxin-response genes involves the interaction of negative regulator Aux/IAA proteins with transcriptional activators (auxin response factors called ARFs) which bind to auxin response elements (AuxREs). The presence of auxin initiates an interaction between a transport inhibitory receptor (TIR1) and Aux/IAA proteins. This interaction causes Aux/IAA proteins to be degraded therefore releasing ARFs to bind to AuxREs in the promoter regions of auxin responsive genes. AuxREs are identified by a short sequence, $^{5'}$TGTCTC$^{3'}$ (Ulmasov et al., 1999) and can bind specifically to ARF homodimers in the promoters of primary auxin-response genes. Once this ARF binding occurs, transcription is activated, resulting in increased gene expression. However, when the ARFs bind to the AuxREs in the promoters as heterodimers with auxin-induced proteins (Aux/IAA), they inhibit gene transcription, thus preventing expression of auxin response genes (Figure 4).

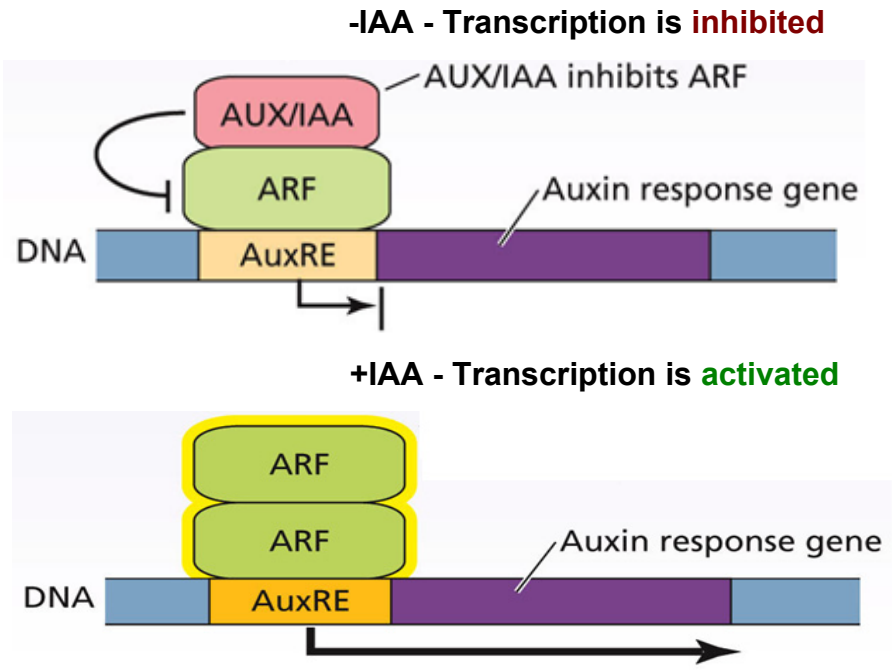


Figure 4. Auxin-response factors (ARFs) bind specifically to auxin response elements (AuxREs) to activate transcription. Auxin-response factors (ARFs) bind specifically to AuxREs in the promoters of primary auxin-response genes. When auxin is absent auxin-induced proteins (Aux/IAA proteins) bind to ARF forming a heterodimer and inhibit transcription of auxin response genes. When auxin accumulates, auxin targets the Aux/IAA proteins for degradation, freeing ARF so it can dimerize. Active ARF dimers activate transcription of auxin response genes.

Plant growth corresponds to the increase in size of pre-existing or newly formed organs and results largely from cell enlargement or elongation. Auxin's primary response is to stimulate cell elongation in most plant tissues. Auxin regulates cell growth by controlling gene expression of auxin response genes by targeting the Aux/IAA proteins for degradation so that auxin response genes can be expressed and regulate responses such as cell wall expansion, enzyme activation, and increased protein synthesis.

Studies show that when plants were treated with 100 μM IAA, ethylene production more than quadrupled within the first two hours demonstrating an interaction between these two hormones. Therefore, the auxin response genes may include

ethylene biosynthetic genes, which may have an auxin response element in their promoter regions. Therefore, increased auxin would result in the activation of the ethylene biosynthesis pathway. Also, even without exogenous application of auxin, ethylene production is increased when plants are reoriented and auxin accumulates on the lower side of a plant (Steed et al., 2004). If a plant is reorienting, the cells on the lower side of the stem elongate to promote upward curvature in stems. Increased auxin will activate gene expression of these auxin-regulated genes.

Hormonal Cross talk between Auxin and Ethylene.

Cell elongation is a common growth and development event controlled by both hormones. Therefore, it is important to discuss how these hormones interact in controlling cell elongation. Auxin-regulated ethylene production is one of the best known hormone interactions in plant biology. Over the last several years, physiological and genetic studies have shown that the role of hormones in plants is determined by complex interactions between hormonal signaling pathways. Researchers have identified molecular components of hormonal metabolism and signaling through many genetic approaches that have been successful in providing a basic molecular understanding of how hormones act within the plant; in particular, hormones auxin and ethylene. Auxin-ethylene cross talk models indicate that increased auxin acts to increase the production of transcription factors that control *ACS* expression (Harrison, 2007). The proposed model suggests how the cross talk between two hormones could potentially regulate a plant growth response in gravitropism (Figure 5). In an *Arabidopsis* seedling, horizontal placement results in asymmetric auxin accumulation in

stem cells, stimulating cell growth on the lower side which may ultimately activate ethylene production by increasing *ACS* expression. In this model of the cell, auxin accumulation, in response to a change in orientation, drives increased ethylene production at the transcriptional level. Here, the regulation of *ACS*, an auxin-response gene, involves the interaction of ARF binding to an AuxRE in the promoter region of the *ACS* gene. Once ARF binding occurs, the *ACS* gene is expressed. However, under low auxin conditions, the ARF-Aux/IAA heterodimer binds to an AuxRE element in the *ACS* promoter and inhibits *ACS* expression. Since the ACS enzyme regulates the rate controlling step of ethylene biosynthesis, this type of inhibition would result in lower ethylene production.

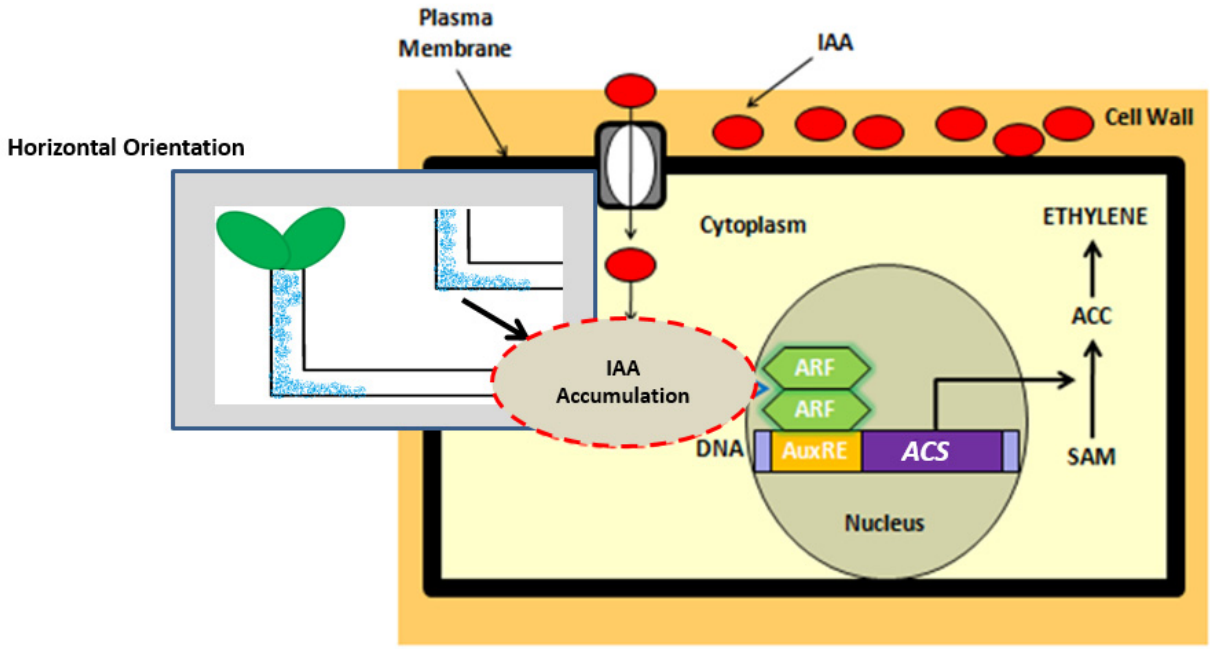


Figure 5. Model of cross talk between two hormones potentially regulating a plant growth response in gravitropism.

One proposed method of evaluating potential cross talk between these two hormones is by evaluating the regulation of *ACS* and *ARF* genes at the transcriptional

level and secondly, how they interact to regulate a response. Therefore, this thesis focused on developing a plant model to evaluate cross talk between ARF and *ACS* in *Arabidopsis* seedlings. The major research objectives were:

1. To determine whether auxin and ethylene interact at the transcriptional level.
2. To determine whether auxin and ethylene regulate specific plant developmental and environmental responses through *ACS* and *ARF* expression changes.

CHAPTER 2:

MATERIALS AND METHODS

Bioinformatical Analysis

Gene loci for each family member were evaluated for the eight functional *ACS* family members and 23 functional *ARF* family members through the TAIR database. Using TAIR's annotated locus details, members of both families were evaluated based upon their tissue localization, changes during development, response to environmental conditions, and/or response to hormone stimulus as indicated in the databases. Published research studies were also used to evaluate tissue expression patterns (Tsuchisaka and Theologis, 2004a) and regulation of the *ACS* genes (Swarbreck et al., 2008). Likewise, experimental evidence was used to indicate the expression of the *ARF* genes (Guilfoyle and Hagen, 2007; Winter et al., 2011) and transcriptional regulation of the *ARF* genes (Okushima et al., 2005; Guilfoyle and Hagen, 2007).

In order to retrieve all promoter sequences and to identify potential ethylene and auxin response elements, locus ID's of promoter sequences for each *ARF* and *ACS*

genes were selected through the TAIR database. The potential plant regulatory elements were identified using the Plant Promoter and Regulatory Element Resources: PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002), Place (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999), and The Arabidopsis Gene Regulatory Information Server (AGRIS) AtcisDB, the *Arabidopsis* cis-regulatory element database (http://arabidopsis.med.ohio-state.edu/AtcisDB/) (Davuluri et al., 2003) databases. Once identified, specific *ARF* candidates and *ACS* candidate genes were selected based on evidence of cross talk by the presence of ethylene response elements (EREs) and auxin response elements (AuxREs) in their promoter regions. EREs were identified by the short sequence, $^{5'}$TTCAAA$^{3'}$ (Oñate-Sánchez and Singh, 2002) and AuxREs were identified by the short sequence, $^{5'}$TGTCTC$^{3'}$ (Ulmasov et al., 1999). The *ARF* and *ACS* candidates were also reported to be involved in gravitropism and/or response to hormone stimulus and expression in the shoots (Swarbreck et al., 2008).

Based on evaluation of gene function, six *ACS* candidates (*ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS8*, and *ACS11*) (Table 1 in Results) and three potential *ARF* candidates (*ARF7*, *ARF11*, and *ARF12*) (Table 2 in Results) were identified as candidate genes to explore ethylene-auxin cross talk. Further discussion for promoter analysis for *ACS* and *ARF* genes will be provided in the Results section.

Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) wild type Col-1 (seed stock CS3176), mutant, and transgenic lines were obtained from The Arabidopsis Biological Resource Center

(ABRC) (Columbus, OH). The *arf7* (seed stock CS24607) mutant is a homozygous recessive loss-of-function mutant generated by a T-DNA (transfer-DNA of the tumor-inducing plasmid of *Agrobacterium tumefaciens*) insertion which disrupts the gene coding sequence. Transgenic seeds carrying *ACS5* promoters fused to GUS reporters were available from the ABRC as seed stock CS31382 developed by Tsuchisaka and Theologis (Tsuchisaka and Theologis, 2004a). All mutant and wild type plants used were in the *Arabidopsis* Columbia ecotype background.

Surface sterilization of seeds began with an initial ethanol wash, followed by three rinses with sterile deionized water, incubated in a mixture of 0.1% TWEEN 80 and commercial bleach (6% sodium hypochlorite) for 5 minutes, then rinsed five times in sterile deionized water. The surface-sterilized seeds were spread on 1.0% (w/v) agar that included half-strength Murashige-Skoog basal salts (0.5 X MS) (Sigma-Aldrich, St. Louis, MO) in square polystyrene gridded Petri dishes (100 x 100 x 15 mm). Seeds were placed next to an area where a 1-2 cm strip of the agar had been removed, so that stems of the germinating seeds would grow above the agar. For growing plants in pots, surface-sterilized seeds were placed in heat-sterilized *Arabidopsis* growth medium (LEHLE SEEDS, Round Rock, Texas). Plates or pots with seeds were cold-treated for 3 days at 4°C. A subsequent one-hour treatment with white light from Phillips flood R4 fluorescent bulbs (average of 75 μmol m^2 s^{-1}) was used to initiate germination. For dark-grown seedlings, the plates were wrapped in foil and oriented vertically in a dark incubator at 23-25^{o}C for three days. This orientation allowed the hypocotyls to grow freely into the open area where the strip of agar had been removed, which was now above them.

Ethylene Analysis

For ethylene analysis, whole plants were incubated in 2-mL shell vials containing seedlings (0.04 g fresh weight each) that were capped with rubber septa. Following a 60 and 120 min incubation times at room temperature, a 1.0 mL headspace sample was injected onto an alumina F1 column (0.635 cm X 0.91 m) in a gas chromatograph (Varian 3700, Varian Instrument Division, Walnut Creek, CA) equipped with a flame ionization detector, according to the equipment parameters previously described (Harrison, 1997). Known amounts of an ethylene standard (Scott Specialty Gases, Plumsteadville, PA) were analyzed to produce a standard curve for ethylene quantitation.

Cross pollination of *arf7* and *ACS5promoter::GUS* plants

The goal of this research was to produce a dihybrid plant that has the promoter reporter construct for *ACS5 promoter::GUS* (*ACS5p*::*GUS*) but lacks the production of ARF7 (the *arf7* mutant). This cross would contain a genotype of homozygous recessive knock-out alleles of ARF7 (designated as recessive genes aa) and homozygous dominant for *ACS5p::GUS* (designated as dominant genes BB).

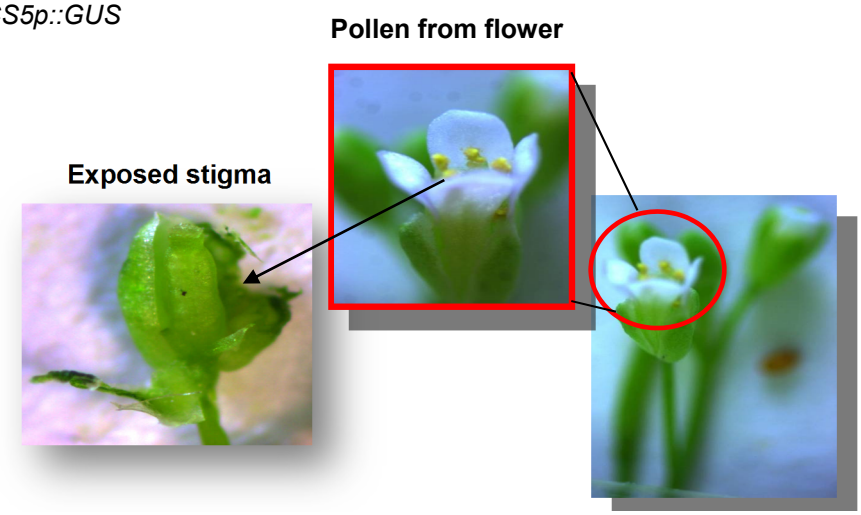
The production of the cross is outlined in Figure 6. The genetic makeup of the parent plant carrying the *ACS5p::GUS* construct is homozygous dominant for both the *ACS5p::GUS* and *ARF* genes, indicated by AABB, where “AA” is +*ARF* and “BB” is +*GUS*. The genetic makeup of the parent *arf7* is *homozygous recessive*, aabb, where “aa” is -*ARF* and “bb” is -*ACS5p::GUS*, since this is a transgene and not found in wild

type plants. Pollen from the anther of an *ACS5p::GUS* plant was transferred onto the stigma of an *arf7* mutant. Sexual reproduction of this cross was to produce the expected genetic offspring of AaBb (F_1 generation), where there was +*ARF* / +*GUS*. To confirm this, the F_1 seeds were planted in soil to later test for *GUS* expression.

The F_1 generation plants were allowed to self-pollinate to produce seeds (F2 generation). The seedlings from the F_2 generation were analyzed for the presence of the GUS reporter gene expression assay to identify plants carrying the *ACS5p::GUS* insert, and homozygous *arf7* mutants. The *arf7* mutants were first characterized by their auxin-resistant phenotypes, then by polymerase chain reaction (PCR) for the presence of the T-DNA insertion.

Alleles

AA = *+ARF7*
aa = *-ARF7*
BB = *+ACS5p::GUS*
bb = *-ACS5p::GUS*



arf7
aabb
(Homozygous Recessive)

ACS5promoter::GUS
AABB
(Homozygous Dominant)

AaBb (F_1 generation)

AaBb x AaBb (F_2 Generation)

	AB	**Ab**	**aB**	**ab**
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	aaBb
ab	AaBb	Aabb	aaBb	aabb

+*GUS* (alleles BB or Bb) screening (indicative by wound response)

Homozygous *arf* (allele aa) phenotypic screening (*arf* mutants grown on IAA agar plates show an altered growth response

Figure 6. Diagram of crosses used to generate plants which are homozygous recessive for *arf7* and contain at least one copy of the *ACS5p*::*GUS* construct.

GUS Reporter Gene Assay

Plants carrying *ACS5p::GUS* fusion were identified by a GUS expression assay (Jefferson et al., 1987). For this assay, entire seedlings or plant tissue were placed in an X-Gluc reaction buffer consisting of the GUS enzyme substrate 2 mM 5-bromo-4-chloro-3-indolyl-β-D-gluclonide (Sigma-Aldrich, Saint Louis, MO) dissolved in 1% (w/v) N,N-dimethyl formamide, with 50 mM sodium phosphate buffer pH 7.0, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide, and 1mM ethylenediaminetetraacetic acid. Seedlings were incubated at 37° C for 4 hours. After incubation, plants were placed in 70% ethanol for clearing and storage. Plants were then imaged on a dissecting microscope with a digital camera (CC-12 Digital color camera, Olympus, Center Valley, PA) for detection of GUS expression as indicated by the blue product of the enzymatic reaction.

Plants carrying the *ACS5p::GUS* fusion were evaluated for wounding-stimulated *ACS5* expression. For this assay, plant tissue was wounded by small punctures or cuts by a fine point forceps. *ACS5* expression during gravitropism was evaluated in horizontally-oriented plants carrying the *ACS5p::GUS* fusion.

Identification and analysis of putative *arf7* mutants

Initially seedlings were screened for auxin resistance, which is a phenotypic characteristic of the *arf7* mutation. This characterization was based on results from a 2006 study that showed when dark grown *arf7* seedlings were grown on various concentrations of IAA, they showed resistance in relative root growth (Li et al., 2006). Their data show that relative root length begins to decrease substantially at IAA

concentrations of 2.5 μM and 5 μM (Li et al., 2006). In addition, *arf7* mutants were resistant to treatment with 1 μM or 5 μM of the ethylene precursor ACC (Li et al., 2006). This auxin-resistance phenotypic screen was also used to analyze F_2 seedlings grown on agar plates that contained 2.5 μM or 5.0 μM IAA.

Plants which exhibited altered response to IAA, were then tested for the presence of T-DNA insertions by PCR amplification using primers for the T-DNA left border (LBb1 of pBIN-pROK2) and gene-specific flanking regions of the insertion, according to the procedure described at T-DNA Primer Design from The Salk Institute Genome Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html) (Alonso et al., 2003). From this information, T-DNA insertion locations were predicted to occur within an exon of the *ARF7* gene. Primer sequences used for identification of homozygousity were primers for the *ARF7* gene: $^{5'}$TGCACTCCTCTTTGAACCATC$^{3'}$ and $^{5'}$CACGAGTTCTTGTTTTAGCCG$^{3'}$, and T-DNA left border sequence LBb1 primer: $^{5'}$GCGTGGACCGCTTGCTGCAACT$^{3'}$. Genomic DNA was extracted from 40 mg of three-week-old *Arabidopsis* rosette leaves (Wizard®Genomic DNA Purification Kit, Promega Corporation, Madison, WI), and sequences were analyzed by PCR according to the HotStarTaq PCR protocol (Qiagen, Valencia, CA). This PCR procedure consisted of a 25 μL reaction mixture typically containing <1 μg DNA, 12.5 μl of 2x HotStarTaq Master Mix, and 2.5 μl of each primer to a 1 μM final primer concentration. The PCR program was run at one cycle at 95°C for 15 minutes, followed by 35 cycles of 30 seconds at 94°C, 60°C for 30 seconds, and 72°C for a one minute final extension, followed by a final soak at 4°C for at least one hour. PCR products were separated by electrophoresis on a 1.0% (w/v) gel (NuSeive 3:1 agarose, Cambrex BioScience,

Rockland, ME) and stained with ethidium bromide. Gels were imaged on a GelDoc 2000 (Bio-Rad Laboratories, Hercules, CA). Predicted product size using the left and right primer for the *ACS7* gene was 1172 base pairs, and the product size of the T-DNA primer (LBb1) and the *ARF7* right primer was estimated to be 600-900 base pairs.

CHAPTER 3:

RESULTS

Locus Analysis for *ACS* and *ARF* genes

The ACS enzyme is encoded by a family of related genes. Eight functional ACS family members are defined by their ACC synthase activity (Table 1). *ACS1* is a pseudogene, but its product has been found to interact with other ACS proteins forming heterodimers (Tsuchisaka and Theologis, 2004b). Most *ACS* genes are also known to be regulated by auxin, especially within the stem and root tips of a plant, with the exception of *ACS1* and *ACS9*, which are not auxin responsive (Table 1). Ethylene production stimulated by mechanical wounding is a major ethylene function, which is caused by increased *ACS* expression. This wounding response is found throughout the lower basal part of the hypocotyls for all *ACS* forms except *ACS9* (Tsuchisaka and Theologis, 2004a).

Multiple members of the ACS family are found in specific plant tissues and many forms act redundantly. For example, many *ACS* forms are expressed in the flower particularly in the pedicel, stamen filament and sepals (*ACS1, ACS2, ACS4*, and *ACS5*) (Table 1). Expression in floral tissue is localized in the silique (seed pod) including the abscission zone (area where the fruit will fall from the stalk), valve (outer walls of the

ovary), and replum (the wall separating the two chambers of seeds) has been reported for *ACS1, ACS2, ACS4, ACS5, ACS6, ACS7, ACS8*, and highly localized in *ACS9*. However, *ACS11* is not expressed in the flower. Many *ACS* forms are co-expressed in the cotyledons, shoot system, and stem (*ACS5, ACS7*, and *ACS11*). In dark-grown seedlings, strong expression is seen throughout the elongation zone of the hypocotyl in *ACS* members *ACS2, ACS4, ACS6, ACS7, ACS8,* and *ACS11*; however, expression is weaker for *ACS1* and *ACS5*, while dark-grown seedlings lack *ACS9* expression. In light-grown seedlings, *ACS* expression exhibits a different localization pattern in the hypocotyl. In this case, stronger expression is seen within *ACS1* and *ACS5* whereas there is no expression in *ACS* members *ACS2, ACS4, ACS6, ACS7, ACS8,* and *ACS11* and very little *ACS9* expression (Tsuchisaka and Theologis, 2004a). The *ACS2, ACS4, ACS5, ACS6, ACS7*, and *ACS8* forms are also co-expressed in young rosette leaves.

Based on data, six *ACS* candidate genes (*ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS8*, and *ACS11*) were identified as of interest in terms of tissue localization and response to auxin (Table 1). The *ACS5* gene was of particular interest because it is an auxin responsive gene and is also wound inducible. In addition, transgenic *Arabidopsis* plants containing promoter regions and the reporter gene GUS are available for all forms of the *ACS* gene family (Tsuchisaka and Theologis, 2004a). Therefore, increased expression of *ACS5* can be documented experimentally by evaluating *GUS* expression in these plants.

The *ARF* genes comprise a large family of 23 genes. Expression patterns within the *ARF* family show similarities to the *ACS* family in that many forms act redundantly in specific tissue types. Many are localized within the hypocotyls (*ARF3, ARF4, ARF6,*

ARF7, ARF10, ARF13, ARF17, ARF19, and *ARF21*) (Table 2) and cotyledons (*ARF7, ARF9, ARF12, ARF17, ARF19*). Also, many *ARF* forms localize to the flower particularly in the stamen (*ARF5, ARF13, ARF17,* and *ARF19*) and the carpel (*ARF2, ARF5, ARF6, ARF7, ARF8, ARF9, ARF10, ARF18, ARF19,* and *ARF21*).

While ARF proteins are activated in the presence of auxin, some are also regulated at the transcriptional level by auxin. Select *ARF* members' expression response are regulated by auxin as well (*ARF4, ARF5, ARF16,* and *ARF19*) (Table 2).

Some *ARF* family members also show increased expression to ethylene treatment (*ARF7, ARF8, ARF9,* and *ARF19*) (Table 2). Therefore, these are potential *ARF* candidates as players in cross talk with *ACS* (Table 2). *ARF7* is of particular interest because of its tissue expression in the hypocotyl and cotyledons and its response to a plant growth response, gravitropism (Table 2). Since *ARF7* is responsive to both auxin and ethylene and to gravitropism, this suggests that cross talk between two hormones could potentially regulate a plant growth response in gravitropism as proposed by the model depicted in Figure 5.

Table 1. Locus identifications for genes encoding enzymes with 1-aminocyclopropane-1-carboxylate synthase (ACS) activity. The expression of each ACS form is localized within specific tissues and regulated under different conditions or by other hormones.

Gene name	Accession[1] #	Tissue Localization[2] of ACS expression	Regulation[2]
ACS1	AT2G43750	Vascular tissue of younger leaves, flower stem in mature plants, flowers (pedicel, stamen filament, sepals), siliques (abscission zone, valve, replum).	Not regulated by auxin. Regulated by wounding throughout the hypocotyl tissue.
ACS2	AT1G01480	Maturation zone and vascular tissue of roots, younger rosette leaves, flowers (pedicel, stamen filament, anthers, sepals), siliques (abscission zone, valve).	Regulated by auxin and expressed constitutively in the root tip. Wounding response occurs only at the basal part of the hypocotyls tissue.
ACS4	AT2G22810	Maturation zone and vascular tissue of roots, younger rosette leaves, flowers (pedicel, stamen filament, sepals), siliques (abscission zone valve).	Regulated by auxin and expressed constitutively in the root tip. Wounding response occurs only at the basal part of the hypocotyls tissue.
ACS5	AT5G65800	Maturation zone and vascular tissue of roots, younger rosette leaves, shoot, cotyledon, stem, flowers (pedicel, stamen filaments, and sepals), style of the silique.	Regulated by auxin and expressed constitutively in the root tip. Has wounding response throughout the hypocotyls tissue.
ACS6	AT4G11280	Maturation zone and vascular tissue of roots, inflorescence stem, siliques, younger rosette leaves, flower (stamen filament, sepals) and silique (abscission zone, valve).	Regulated by auxin and expressed constitutively in the root tip. Wounding response only at the basal part of the hypocotyls tissue.
ACS7	AT4G26200	Cotyledons shoot system, stem, maturation zone and vascular tissue of roots, younger rosette leaves, flowers (stigmatic tissue, stamen filament, anthers, sepals), siliques (abscission zone, valve).	Regulated by auxin and expressed constitutively in the root tip. Auxin has a localized effect on the expression of *ACS7*. Wounding response occurs only at the basal part of the hypocotyl tissue.
ACS8	AT4G37770	Maturation zone and vascular tissue of roots, root cap, younger rosette leaves, flowers (pedicel, stamen filament, anthers, sepals), siliques (abscission zone valve).	Regulated by auxin and expressed constitutively in the root tip. Wounding response occurs only at the basal part of the hypocotyls tissue.
ACS9	AT3G49700	Barely expressed in seedlings. Not expressed in the root. Highly localized expression at the style of the silique of the flower.	Not regulated by auxin. No wounding response in the hypocotyls.
ACS11	AT4G08040	Cotyledon, guard cell, petiole, vascular leaf. Restricted to the trichomes of leaves.	Regulated by auxin and expressed constitutively in the root tip. Wounding response only at the basal part of the hypocotyls tissue.

[1] (Swarbreck et al., 2008)

[2] (Tsuchisaka and Theologis, 2004a)

Table 2. Locus identifications for genes encoding auxin response factor (ARF) which have specific DNA binding transcription factor activity in response to auxin. The expression of each ARF form is localized within specific tissues and regulated under different conditions or by other hormones. No data were available for *ARF14* (At1g35540), *ARF15* (At1g35520), *ARF20* (At1g35240), and *ARF22* (At1g34390).

Gene name	Accession[1] #	Tissue Localization[3,5] of ARF expression	Regulation[3,4]
ARF1	AT1g59750	Flowers, dry seeds, shoot apex, siliques.	Decreased slightly in response to dark-induced senescence in leaves.
ARF2	AT5g62000	Flowers, carpels, dry seed, shoot apex, cauline, floral organs, light-grown and dark-grown seedlings.	Increased moderately in response to dark induced senescence in leaves
ARF3	At2g33860	Flowers, shoot apex, hypocotyl, reproductive and vegetative tissues.	
ARF4	At5g60450	Flowers, shoot apex, stem, hypocotyl, petals, seeds w/o siliques, reproductive and vegetative tissues.	Increased slightly in response to auxin. Expressed during leaf senescence.
ARF5	At1g19850	Flower, stamens, carpels, shoot apex, embryos, vascular tissues.	Increased slightly in response to auxin.
ARF6	At1g30330	Hypocotyl, flowers, carpels, shoot apex.	
ARF7	At5g20730	Flowers, carpels, petals, senescing leaves, rosettes, dry seeds, hypocotyl, cotyledons, roots, seeds w/o siliques.	Increased moderately in response to dark induced senescence in leaves. Involved in gravitropism; responsive to ethylene stimulus
ARF8	AT5g37020	Flowers, carpels, petals, shoot apex, seedlings, developing flower and fruits.	Expression in seedlings increased in response to light. Ethylene responsive.
ARF9	At4g23980	Flowers, carpels, roots, cotyledons, senescing leaves, shoot apex.	Increased moderately in response to dark induced senescence in leaves. Ethylene responsive.
ARF10	At2g28350	Flowers, carpels, petals, senescing leaves, root, hypocotyl, shoot apex, seeds with and w/o siliques.	
ARF12	AT1g34310	Sepals, pedicels, leaves, cotyledons, seeds.	
ARF11	AT2g46530	Seeds with and w/o siliques, shoot apex.	
ARF13	At1g34170	Sepals, stamen, root, seeds w/o siliques, hypocotyl, shot apex.	
ARF16	At4g30080	Flowers, petals, dry seeds, cauline leaves, senescing leaves, root, seeds with and without siliques, basal region of embryos, root caps, vascular tissue of roots, and leaves.	Increased slightly in response to auxin.
ARF17	At1g77850	Flowers, petals, stamen, dry seed, cotyledons, hypocotyl, root, seeds with and without siliques.	
ARF18	At3g61830	Flowers, carpels, dry seed, shoot apex.	
ARF19	At1g19220	Petals, stamen, carpel, flowers, cauline leaves, hypocotyl, cotyledons, shoot apex, seedlings, roots.	Increased slightly in response to auxin. Response to ethylene stimulus.
ARF21	At1g34410	Petals, carpels, hypocotyl, senescing leaves, seeds w/o siliques, shoot apex.	
ARF23	At1g43950	Mature pollen.	

[3] (Guilfoyle and Hagen, 2007)

[4] (Okushima et al., 2005)

[5] (Winter et al., 2011)

Promoter response element analysis for *ACS* and *ARF* genes

Each *ACS* and *ARF* locus was evaluated using databases listed in the TAIR Plant Promoter and Regulatory Element Resources to first identify their predicted promoter sequences (at least 1000 base pair upstream of the transcription start site) and secondly, to locate the presence of the primary EREs and/or AuxREs in the promoter region of *ACS* and *ARF* family members.

PlantCARE, Plant Cis-Acting Regulatory Elements, is a referential database with 435 different names of plant transcription sites from 149 from monocots, 281 from dicots, and five from other plants, describing more than 159 plant promoters (Lescot et al., 2002). PLACE, A Database of Plant Cis-acting Regulatory DNA Elements, provides information extracted from previously published reports for vascular plants only (final update was Jan. 8, 2007) (Higo et al., 1999). AGRIS, The Arabidopsis Gene Regulatory Information Server, is an information resource of *Arabidopsis* promoter sequences, transcription factors and their target genes. AGRIS includes AtcisDB which contains "approximately 33,000 upstream regions of annotated *Arabidopsis* genes (TAIR release) with a description of experimentally validated and predicted cis-regulatory elements" (Davuluri et al., 2003). In contrast to AtcisDB, both PlantCARE and PLACE include numerous plant species and are not limited to *Arabidopsis*.

Using projected promoter sequences for *ACS* forms, PlantCARE identified a response element ($^{5'}$TGTCTC$^{3'}$) for ethylene in the promoter regions of *ACS* family members *ACS2* and *ACS5* (Table 3); whereas the PLACE database also found the same ethylene response element in *ACS2* and *ACS5* but also in *ACS4*, *ACS6*, and *ACS11,* while the AtcisDB database AtcisDB found no ethylene response elements in

the promoter regions of the *ACS* genes (Table 3). However, AtcisDB reveals the presence of the binding site for the ethylene response factor 1 (ERF1), and not the ERE sequence found by PLACE and PlantCARE. The AtcisDB identifies an ERF1 binding site with the GCC box. ERF1 binds and activates numerous secondary ethylene responsive genes, thus indicating another potential mechanism of ethylene self-regulation in *ACS* genes. PLACE identifies the ethylene response element found in *ACS5* as similar to one found in wheat, tobacco, and carnation *ACS* genes. In *ACS8*, there is no ethylene response element mainly because that gene is not regulated by ethylene.

PLACE identifies several auxin responsive element loci in the *ACS5* promoter*, a* single location in the *ACS4* promoter area, and two loci in the promoters of *ACS6*, *ACS8*, and *ACS11* (Table 3*)*. The AtcisDB database finds multiple notations of an auxin response element in *ACS5*, as well as a single notation in *ACS4*, and double notations in *ACS6*, *8*, and *ACS11* (Table 3).

Selected *ACS* family members, *ACS2*, *ACS4*, *ACS5*, *ACS6*, and *ACS11*, have both auxin and ethylene response elements in their promoter regions (Table 3). Promoter analysis showed that *ACS5* is also an auxin responsive gene that has both an auxin and ethylene response element in its promoter region (Table 3).

PlantCARE only located one copy of the response element is for ethylene in the promoter regions from specific *ARF* family members, *ARF3, ARF6, ARF9,* and *ARF12* (Table 4); whereas PLACE, also found the same ethylene response element in all *ARF* family members except for *ARF19* (Table 4). In contrast, the AtcisDB database did not identify any EREs in the *ARF* genes searched, but did identify a binding ERF1 in the

ARF7 promoter. PLACE identifies several areas where there seems to be multiple auxin responsive elements in *ARF3, ARF20, ARF10,* and *ARF19*; double copies in *ARF9* and *ARF11*; and a single copy in *ARF1, ARF4, ARF6, ARF12, ARF15,* and *ARF21.* The AtcisDB database confirms the auxin response elements found in PLACE.

The data also show that the promoter regions of *ARF11* and *ARF12* contain both auxin and ethylene response elements (Table 4). However, in the *ARF7* promoter, there is no auxin response element site because that gene is not regulated by auxin at the transcriptional level. Promoter analysis showed that *ARF7* has an ethylene response element in its promoter region. Since *ARF7* is responsive to two hormone stimuli (auxin and ethylene) and to a plant growth response (gravitropism), evidence suggests that cross talk between two hormones could potentially regulate a plant growth response in gravitropism.

Table 3. The number of promoter response elements identified for *ACS* genes.

	ERE site			**AuxRE site**		
	AtcisDB	**PlantCARE**	**PLACE**	**AtcisDB**	**PlantCARE**	**PLACE**
ACS2	0	2	3	0	1	0
ACS4	0	0	1	1	1	1
ACS5	0	1	1	4	1	3
ACS6	0	0	5	3	0	2
ACS7	0	0	0	0	0	0
ACS9	0	0	0	2	0	2
ACS11	0	0	1	2	0	2

Table 4. The number of promoter response elements identified for *ARF* genes. Data was not available for *ARF* genes (*ARF2, ARF5, ARF8, ARF13, ARFs 16-18, ARF22,* and *ARF23*).

	ERE site			AuxRE site		
	AtcisDB	**PlantCARE**	**PLACE**	**AtcisDB**	**PlantCARE**	**PLACE**
ARF1	0	0	1	1	0	1
ARF3	0	2	4	4	0	4
ARF4	0	0	1	1	1	1
ARF6	0	2	3	1	2	1
ARF7	0	0	1	0	0	0
ARF9	0	2	3	2	1	2
ARF10	0	0	2	3	0	3
ARF11	0	0	1	2	1	2
ARF12	0	1	1	1	0	1
ARF14	0	0	2	0	1	0
ARF15	0	0	1	1	0	1
ARF19	0	0	0	3	1	3
ARF20	0	0	2	4	4	4
ARF21	0	0	1	1	0	1

Phenotypic screen for aaBB and/or aaBb (*-ARF*, *+ACS5p::GUS*) mutants

The F_1 plants resulting from AABB x aabb produced offspring (AaBb), which is heterozygous for both genes *+ARF*/ *+GUS*. To confirm this, the F_1 seeds were planted

in soil to later test for *GUS* expression. The expression of *ACS5p::GUS* was verified in F_1 generation the by its strong wounding response that shows a strong blue color (*GUS* expression) in the leaf tissue at the wounded cut and needle puncture sites (Figure 7a). While the AaBb mutants have only one copy of the construct, they showed a somewhat diminished wounding response, but still clearly exhibit *GUS* expression in the leaf tissue (Figure 7b). Since these plants were heterozygous for *ARF*, they behaved phenotypically like wild type, so could not be screened for phenotypic *arf* mutant characteristics.

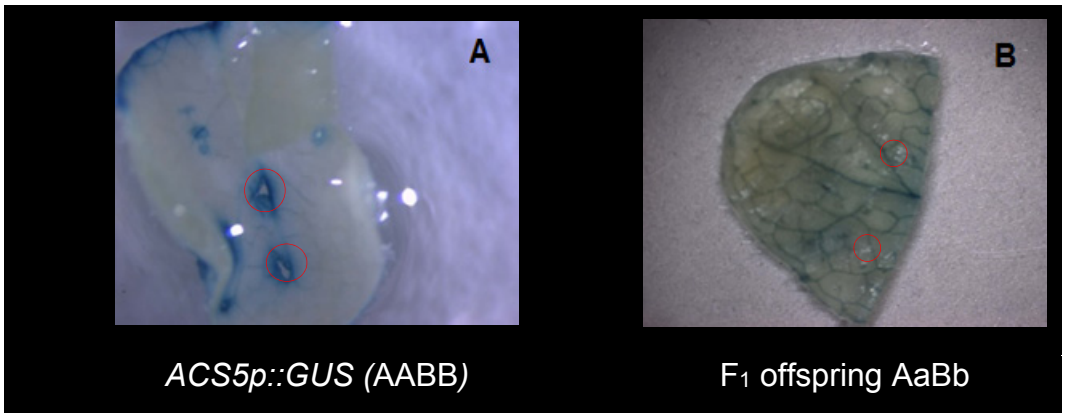


Figure 7. *GUS* expression in leaves of plants homozygous for the *ACS5p::GUS* construct and the heterozygous F1 generation. Circles indicate areas of wounding.

The F_1 generation (AaBb) was allowed to self-cross, thus passing on a combination of alleles (aaBB and aaBb) to the F_2 generation. Selection for the homozygous *arf7* alleles was through evaluation of their phenotypic response to auxin. For this, dark-grown F_2 seedlings grown on IAA concentrations of 2.5 µM and 5.0 µM which showed growth inhibition compared to that of wild type were considered to be homozygous for *arf7* (Figure 8a and b.), consistent with the data presented by Li et al. (2006). These selected F_2 seedlings also showed irregular orientation compared to wild

type, indicating altered auxin response in gravitropism, also a characteristic of *arf7* mutants.

The F_2 seedlings identified as homozygous *arf7* mutants were transplanted to soil and allowed to mature. These plants were then screened for *GUS* expression, indicating the presence of the *ACS5p::GUS* construct (Figure 9). Plants containing positive *GUS* expression were then collected as the cross (either Aabb or aabb) for further characterization.

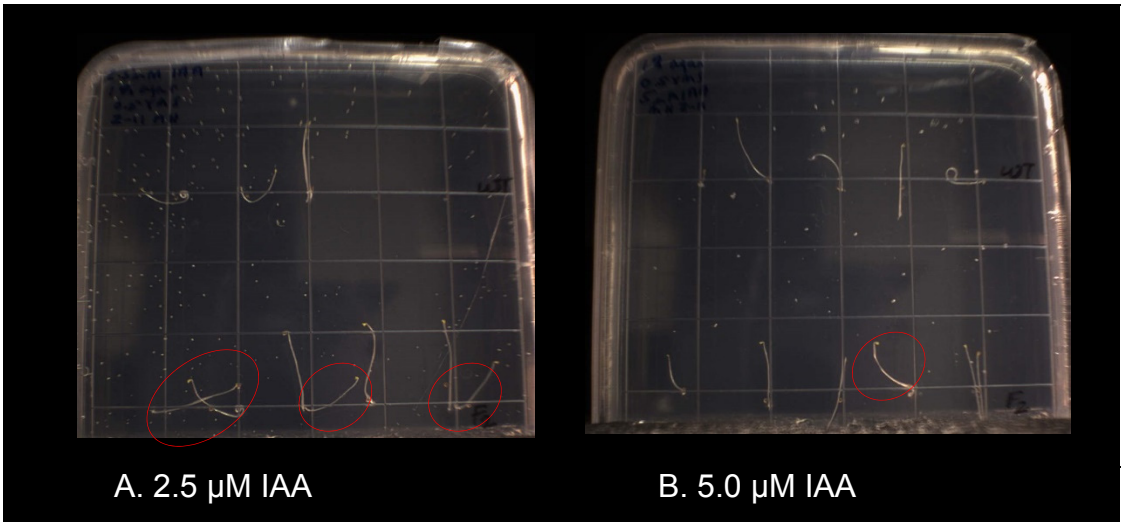


Figure 8. Phenotypic screening of F_2 generation seedlings. Select F_2 seedlings grown on IAA concentrations of 2.5 µM (A) and 5.0 µM (B) showed altered growth in their orientation compared to that of wild type. Circles indicate selected F_2 seedlings.

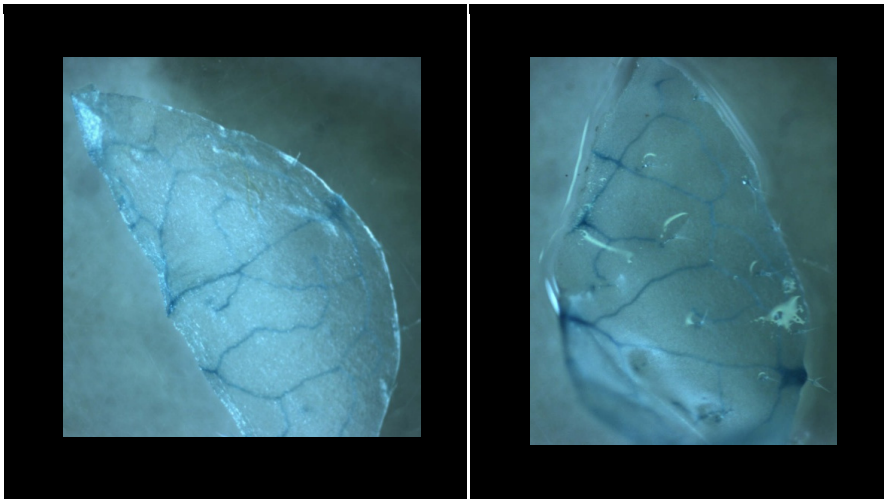


Figure 9. *GUS* analysis for representative F_2 generation dark-grown seedlings which showed growth inhibition when grown on plates containing IAA compared to wild type. Those plants circled in red from Figure 8 were selected to test for GUS activity.

Verification that F_2 plants are homozygous for aaBB and/or aaBb (*-ARF/+ ACS5p::GUS*) genotypes.

Confirmation of the *GUS* reporter gene insertion of the cross pollination of *arf7* and *ACS5p::GUS* has been seen throughout F_1 and F_2 generations. However, thus far, a phenotypic screen of the seedlings of the F_2 generation has only shown that this cross shows some resistance to vertical orientation in the presence of IAA. A more molecular approach to confirm that this cross not only contains the *ACS5p::GUS* construct, it also contains two copies of the T-DNA insert associated with the *arf7* knock-out mutation. Identification of the T-DNA insertion can be identified through PCR using a triplex primer mixture for left and right primers for regions on exons of the *ARF7* gene and also a primer for the left border of the T-DNA insert. PCR amplification of plants containing the T-DNA shows the presence of a smaller DNA band of approximately 600-900 base pairs, which is the amplification of the region identified by the primer for a region on the T-DNA left border primer and the right primer that identifies a region on an *ARF7* exon

(sample A in Figure 10). Wild type plants which do not contain a T-DNA insert have a larger product (>1000 bp), which is the amplification of the *ARF* DNA. Results showed that the genotype of one of these plants (sample A) confirmed to be homozygous for aaBB and/or aaBb (-*ARF*/+ *ACS5p:: GUS*) (Figure 10).

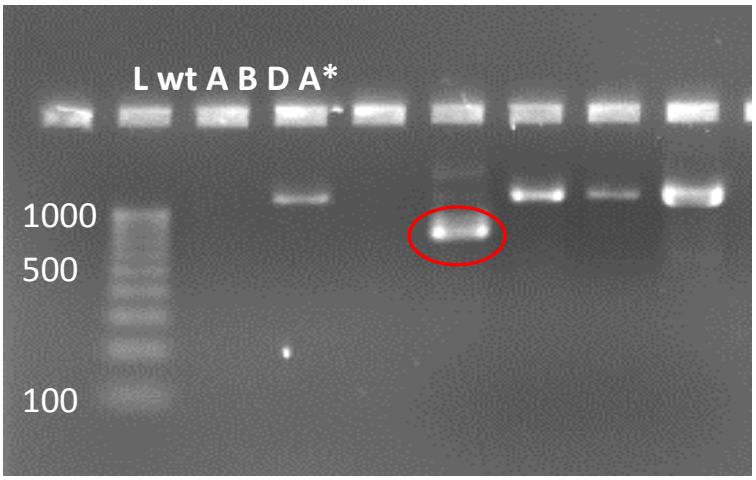


Figure 10. Analysis of *arf7* expression shows a homozygous genotype of aaBB and/or aaBb (-ARF / +*ACS5p::GUS*) in a F_2 generation plant A. An aliquot of 5 ng of DNA from selected seedlings of the F_2 generation was constituted and separated on a gel to detect homozygous inserts. 10 µl samples were loaded per well. Wild type (wt) DNA, potential cross plants (A, C, and D) were amplified with *ARF* right and left primers, and the left border primer (LBP1.3). The A* sample DNA was an additional control that was amplified with only the *ARF* primers. L represents a 100 base pair ladder. The predicted product size of the wild type *ACS7* gene was 1172 base pairs, and the product size of the T-DNA primer (LBb1) and the *ARF7* right primer was estimated to be 600-900 base pairs (circled). Unlabeled lanes were not loaded with sample.

Characterization of cross

Tissue localization of ACS5 expression. Dark-grown *ACS5p::GUS* transgenic seedlings show *ACS5* expression throughout the shoot region, especially in the vascular tissue in the center of the hypocotyl (Figure 11). The immature leaves also show a fair amount of expression. The cross also shows *ACS5* expression, but to a lesser extent. In the cross, there is expression only in the vascular tissue of the leaves and in the upper portion of the hypocotyl. *GUS* expression is diminished in the lower portion of the hypocotyl and in the leaf tissue. Wild type plants and *arf7* do not carry the

ACS5p::GUS construct, and do not exhibit *GUS* expression.

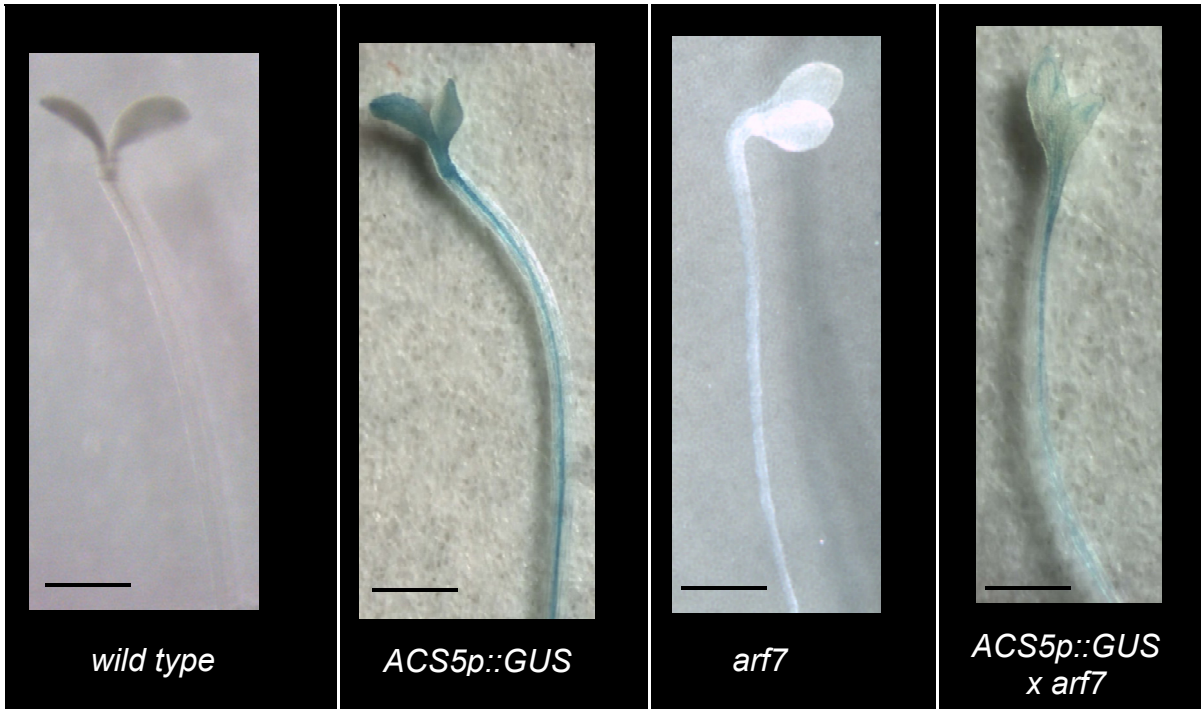


Figure 11. *GUS* expression in dark-grown *Arabidopsis* seedling shoots. Bars = 0.5 mm.

In light grown eight-day-old seedlings, expression is shown in *ACS5p:: GUS* and confirmed in *ACS5p:: GUS x arf7* cross in the veins and cells of the cotyledons and young leaves (Figure 12). No expression of *GUS* is localized in wild type or *arf7* plants which do not carry a *GUS* reporter gene. In the light-grown seedlings, the *ACS5* expression pattern in *ACS5p::GUS* x *arf7* cross is similar to the *ACS5p::GUS* parent plants in the strong expression in the cotyledons and young leaves. Some plants also exhibited *ACS5* expression in the hypocotyl vascular tissue. The vascular tissue of the root shows weak expression in the vascular tissue for both *ACS5p::GUS* x *arf7* (data not shown) and *ACS5p::GUS.*

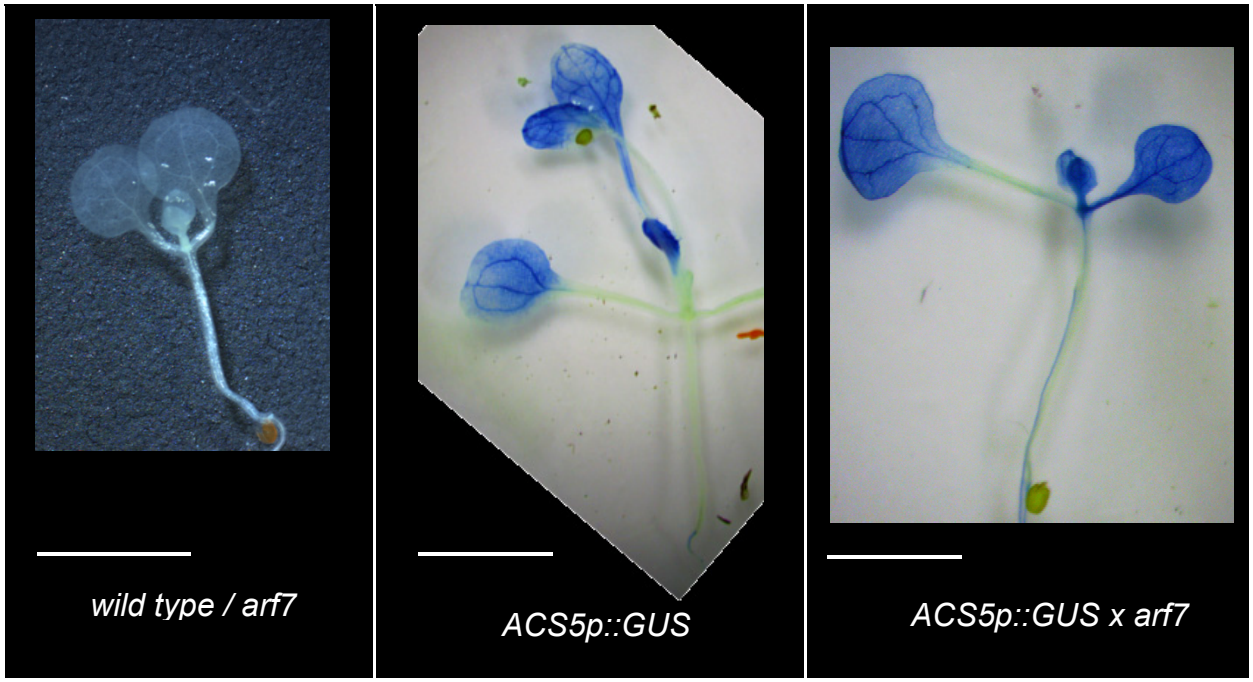


Figure 12. Expression of *GUS* in light-grown seedlings of *Arabidopsis*. *GUS* is expressed in the *ACS5p::GUS* and *ACS5p::GUS x arf7* cross in the apical bud area and cotyledons. Expression is also seen in the hypocotyl and roots of *ACS5p::GUS*. Bars = 1 cm.

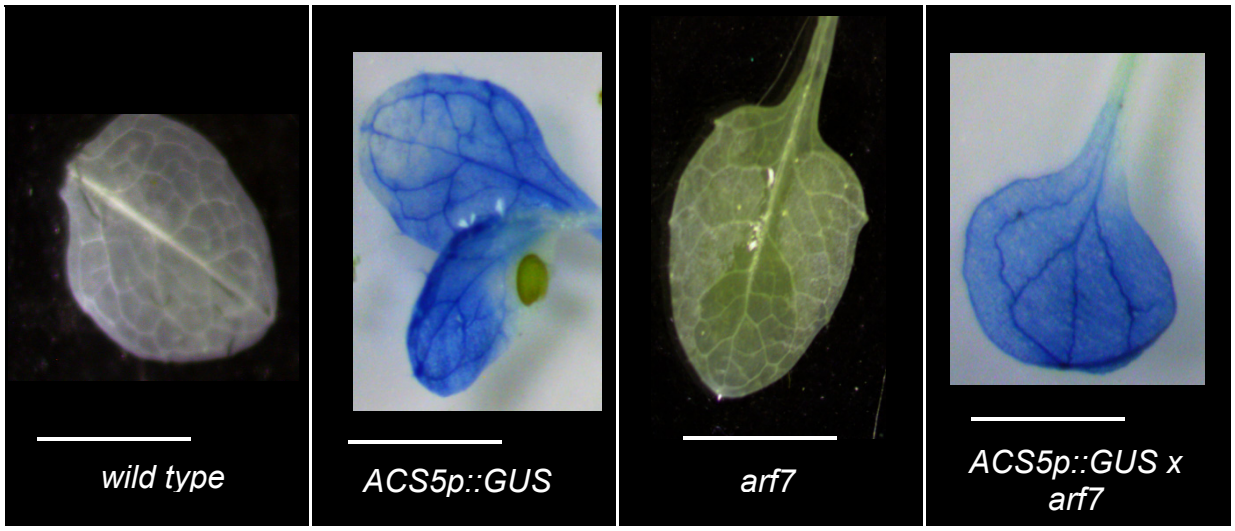


Figure 13. Expression of *GUS* in leaf tissue and in the vascular tissue of both *ACS5p::GUS* and *ACS5p::GUS x arf7* samples. Bars = 0.5 cm.

Expression in flowers. In the flowers of light-grown plants, *ACS5* expression is seen in

the filaments and sepals but not in the anthers of *ACS5p::GUS* plant (Figure 14). In more mature flowers, *ACS5* is expressed in both male and female portions of the plant; filaments and anthers (male portion) and the stamen (female portion) in *ACS5p::GUS* plants (Figure 17c). There was no expression observed in young flowers of the cross and little expression in older tissue (data not shown). In all samples, the cross exhibited less expression in flower structures. The wounding response seen at the point of excision of the peduncle was strong in both *ACS5*p::*GUS* and the cross indicating equal levels of the *ACS5p::GUS* construct in these plants.

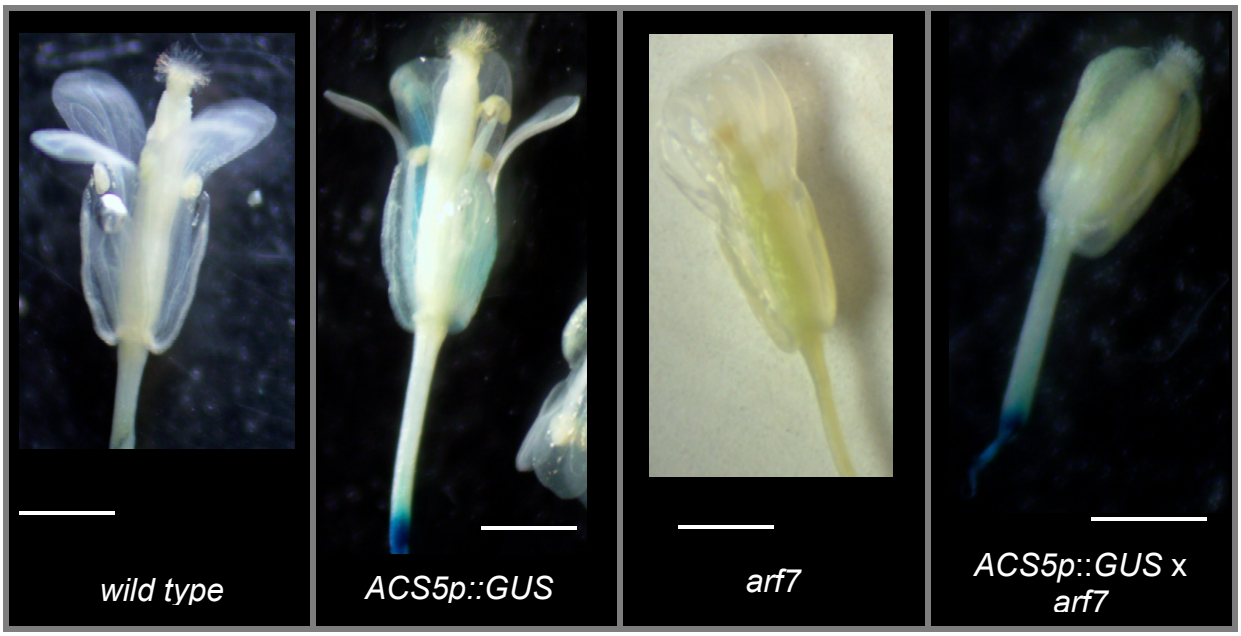


Figure 14. Representative tissue distribution of *ACS5* expression in flower of *ACS5p::GUS* and *ACS5p::GUS x arf7* plants. No ACS5 expression was observed in wild type or arf7 mutants lacking the *ACS5p::GUS* construct. Bars = 1 mm.

Exogenous IAA application stimulates *ACS5* expression in the hypocotyls. In dark grown five-day-old seedlings, exogenous application of 2.5 μM or 5.0 μM IAA agar blocks were placed near hypocotyls to determine the effect on the expression of *ACS5*. IAA blocks induced an area of stronger expression next to the application site (red

arrows) for both concentrations (Figure 13). In contrast, little expression is observed next to the block site for the *ACS5p::GUS x arf7* cross, although these plants show strong expression in the apical area, young cotyledons, and in the vascular tissue. Overall, 5.0 µM IAA concentration shows stronger expression level in the hypocotyl for the *ACS5p::GUS* seedlings.

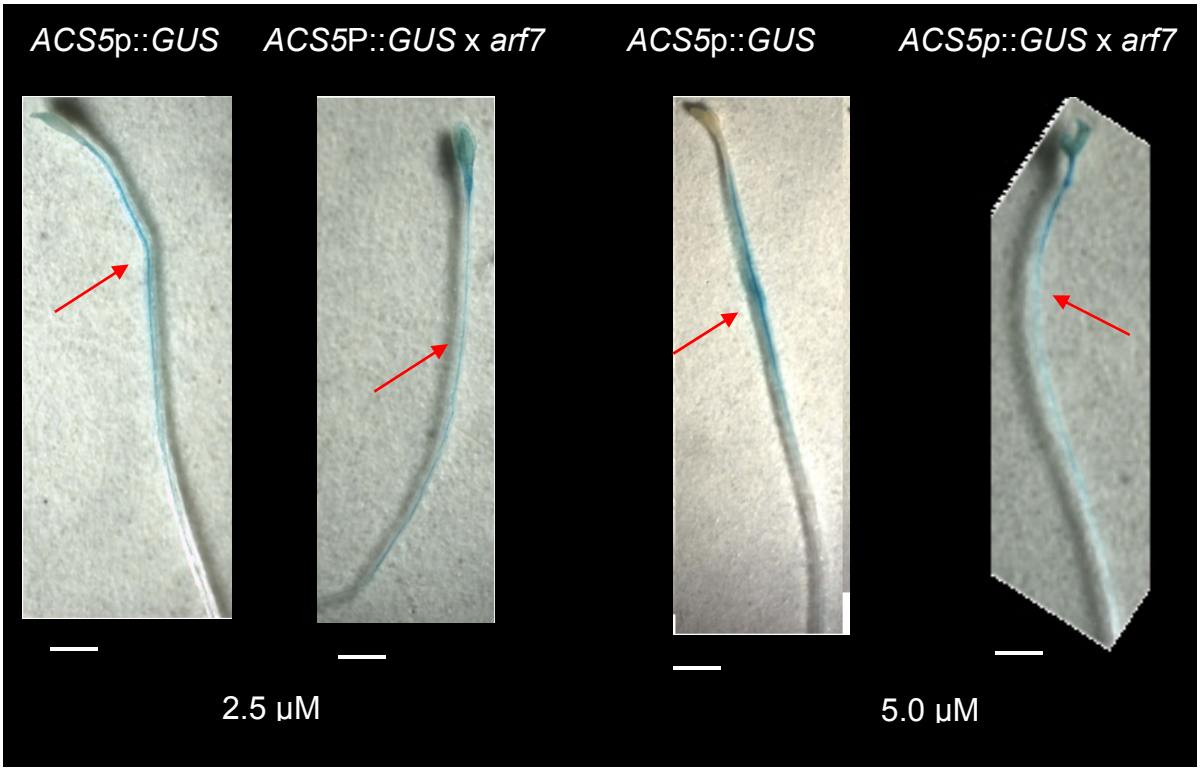


Figure 15. Analysis of localized IAA treatment on five-day-old dark grown *Arabidopsis* seedlings. Red arrows indicate sites of IAA-agar block placement on the hypocotyl. Bars = 0.5 mm.

Gravitropism. When five day-old seedlings were reoriented to a horizontal position, cells elongated to reorient themselves back to their original gravitational field. Vertical position at 0 hours are assumed to have equal distribution of auxin which causes straight growth of the hypocotyl. Both *ACS5p::GUS* and *ACS5p::GUS x arf7* show the equal distribution of *ACS5* expression and straight growth at 0 hours. Horizontal re-

orientation of *ACS5p*::*GUS* and *ACS5p::GUS x arf7* seedlings shows curvature at seven hours in the hypocotyl likely caused by the asymmetric distribution of auxin. However, the increased auxin on one side of the hypocotyl did not result in increased *ACS5* expression for either the *ACS5p*::*GUS* or *ACS5p*::*GUS* x *arf7* seedling although somewhat stronger expression is observed in the hypocotyl tissue at seven hours reorientation.

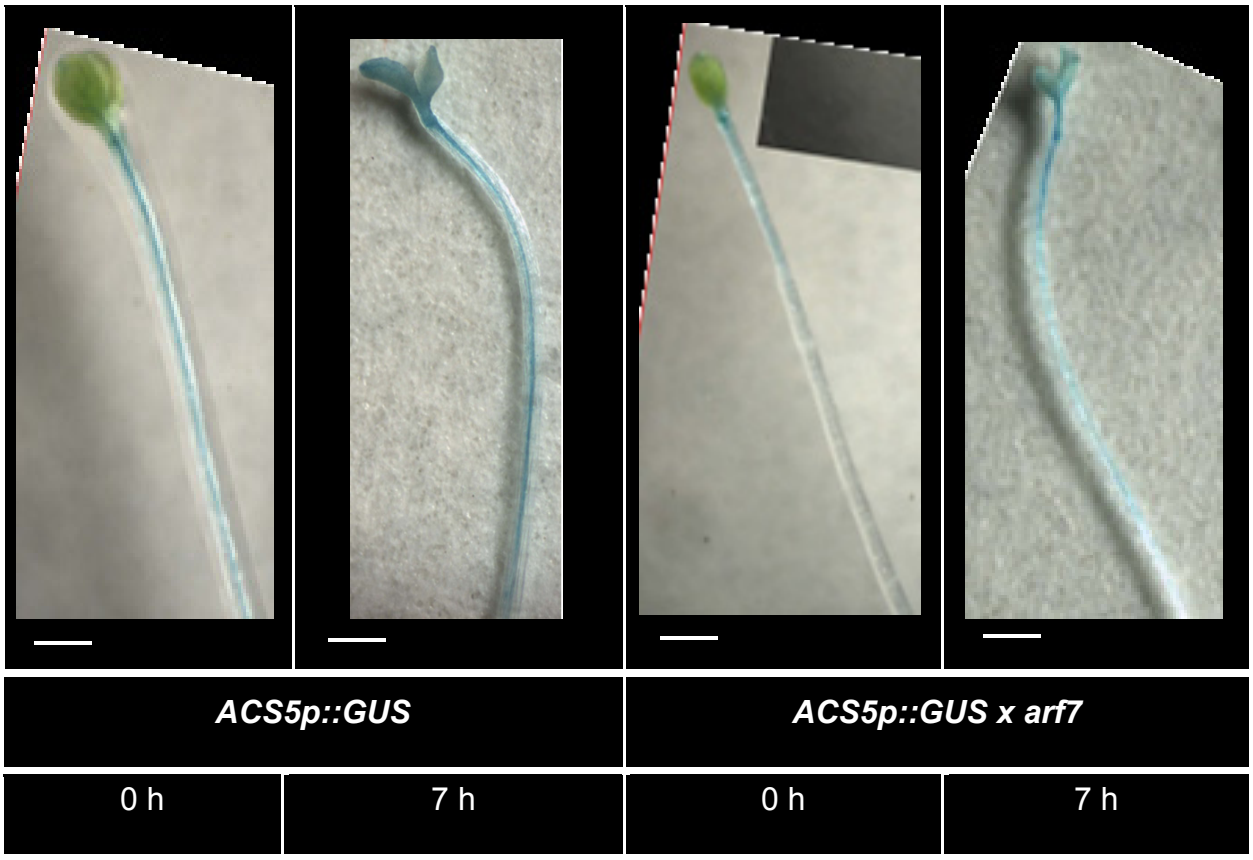


Figure 16. Analysis of *ACS5* expression after seven hours reorientation (gravistimulated) in five-day-old dark grown *Arabidopsis* seedlings. Bars = 0.5 mm.

Wounding. Light-grown plants show a wound-induced expression of *ACS5* in different tissues within the plant (Figure 17). Higher levels of *ACS5* expression are observed in localized regions adjacent to the cut area of the leaf, and at the point of excision of the tissue (excised petiole for leaves and peduncle for flowers). Both *ACS5p::GUS* and the

ACS5p::GUS x arf7 cross plant tissues exhibit equally high levels of wound-induced *ACS5* expression.

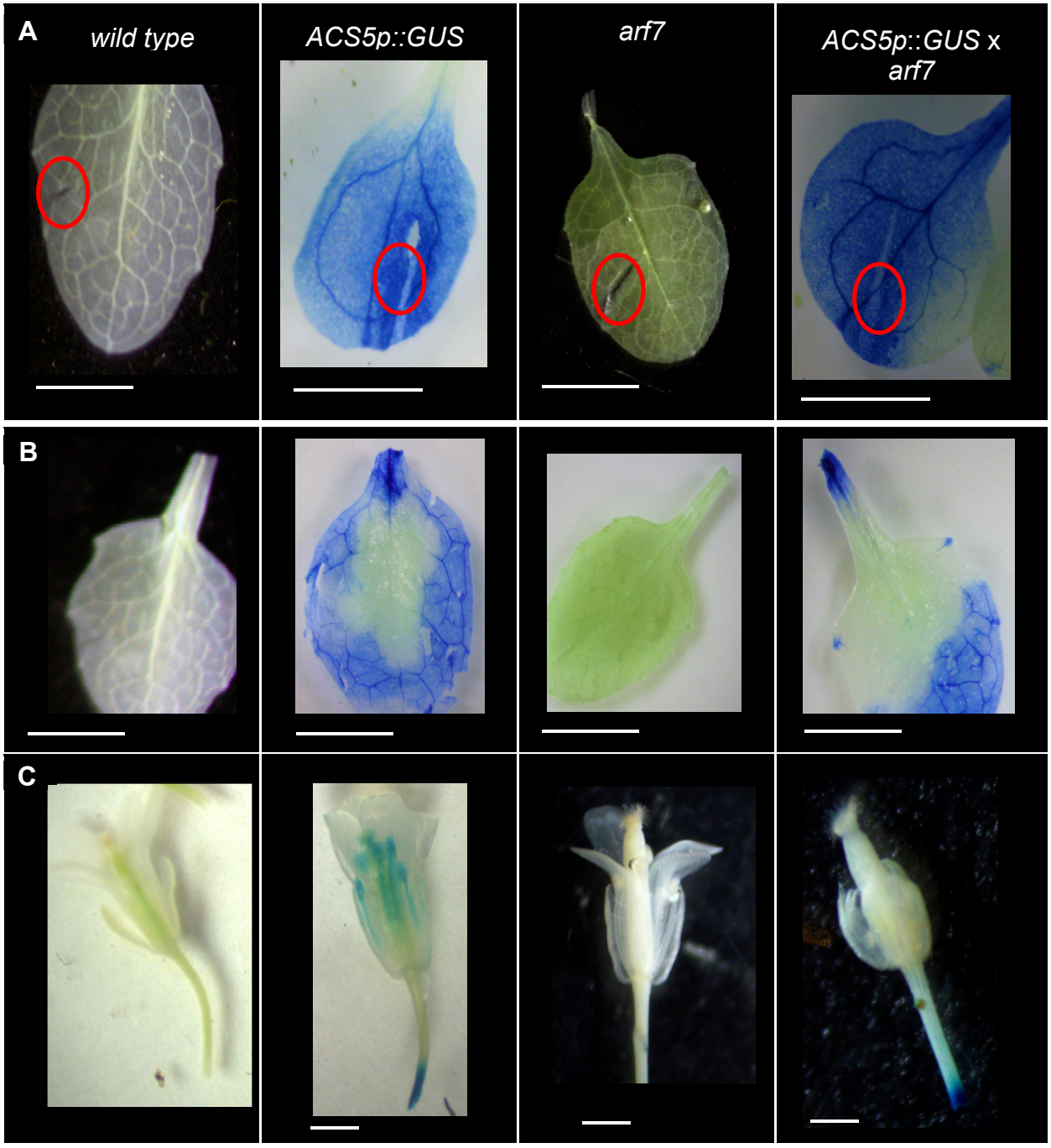


Figure 17. Representative samples for the evaluation of wounding on *ACS5* expression in A) wounded leaf tissue, B) excised areas of the leaf petiole, and C) excised area of the flower peduncle. . The red circles indicate the area of wounding by cutting in young leaves of light-grown *Arabidopsis* seedlings. Bars = 0.5 cm (leaves) and 1.0 mm (flowers).

Ethylene production. Stem and leaf tissue showed ethylene production after 60 and 120 minute incubation in a closed vial (Figure 18). Samples included leaf and stem tissues which were excised to keep all samples the same weight of 0.04 g. Wild type and *ACS5p:: GUS* whole plant samples produced similar amounts of ethylene for the 60-minute incubation, but the *ACS5p::GUS* plant sample showed a decrease in ethylene for the 120 min incubation. The *ACS5p::GUS X arf7* sample produced somewhat less ethylene than wild type and *ACS5p::GUS* but more than *arf7* plants.

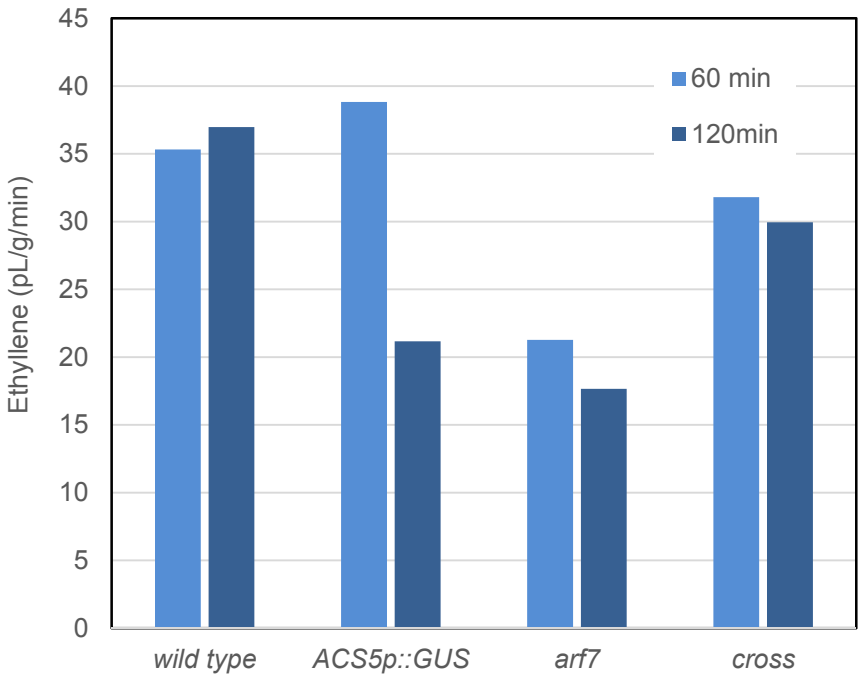


Figure 18. Ethylene production after 60 or 120 minute incubation for leaf and stem tissues in wild type compared to *ACS5p::GUS, arf7,* and *ACS5p::GUS X arf7 (cross)* samples.

This data were used to evaluate a trend in ethylene levels, but because of the amount of tissue required, gas chromatography was only performed once in cross plants. Therefore, the inconsistencies in the data could not be evaluated further. Also, ethylene is produced in the presence of oxygen, so in some cases the oxygen is

depleted in the vials causing ethylene production to decrease. Depleted oxygen levels may contribute to the lower level of ethylene observed during the second incubation time. Therefore, it is interpreted that a more direct approach to measuring ethylene is during the first 60 minutes, since it is assumed that this ethylene is produced prior to the onset of wound-induced ethylene and there is adequate oxygen. Although there are important ethylene production differences seen in various plants, no statistical analysis is possible.

CHAPTER 4:

DISCUSSION

Database analysis

Bioinformatical analysis of *ARF* and *ACS* expression patterns demonstrates that there is overlapping expression in many tissues and under different conditions (Tables 1 and 2). While *ACS5* and *ARF7* genes were chosen for the cross talk investigation, it is recognized that other forms are also present in the tissue. The ACS enzymes primarily function as homodimers but may also form active heterodimers in some conditions (Tsuchisaka and Theologis, 2004b). While many ARF proteins act as homodimers (Boer et al., 2014), ARF7 has been demonstrated to interact with ARF19 in its function forming a heterodimer. ARF19 is known to respond to hormonal activity from auxin and ethylene (Guilfoyle and Hagen, 2007), and bioinformatical analysis indicates ARF19 levels increase slightly in response to auxin and response to ethylene stimulus (Table 1). The ARF7 protein binds to auxin response elements in the promoter region of *ARF19* increasing its transcription, causing a response to both hormones in specific cell

types. *ARF7* and *ARF19* are both regulated in the hypocotyl, cotyledons, roots, and seedlings (Guilfoyle and Hagen, 2007). Double mutants of *ARF7* and *ARF19* (*arf7/arf19*) are extremely auxin and ethylene resistant (Li et al., 2006).

Analysis of the *ACS* and *ARF* promoter regions reveals that multiple forms contain both auxin and ethylene response elements. However, there are differences in the documentation for the presence or absence of the response elements in the *ACS* and *ARF* promoters depending on the type of tools employed (Tables 3 and 4). For example, in the *ARF7* promoter region, PLACE identifies an ethylene response element in its promoter region whereas PlantCARE does not. Factors which contribute to the differences in consistently identifying these response elements include the types of approaches and limitations (e.g., species studied, publication dates) of the different promoter analysis tools. PLACE has not been updated since 2007, while PlantCARE allows users to continually contribute to its database. Both PlantCare and PLACE are not just limited to *Arabidopsis*, whereas AtcisDB defines the 1000 upstream base pair region for the *Arabidopsis* genes and does not allow input of the actual sequence. Therefore, the AtcisDB promoter analysis tool was useful for experimental design, but not comparable to the other tools. Nonetheless, this type of bioinformatical data provided strong evidence that there are EREs and/or AuxREs in the promoter region of *ACS* and *ARF* family members providing evidence of cross talk between hormones auxin and ethylene at the transcriptional level.

Also, it should be noted that this study focused on the presence of EREs sequences which bind to the EIN3 transcription factor as the primary indicator of ethylene cross talk. EIN3 activates the transcription of *ERF1* whose product is

responsible for regulating numerous secondary ethylene response genes. In fact, AtcisDB identifies an ERF1 binding site (the GCC box) and not an ERE in the promoter region of the *ARF7* gene. Therefore, continued analysis for the GCC box could identify further secondary genes involved in cross talk.

Proposed model for *ARF7* and *ACS5* cross talk

Figure 19 provides a model for auxin and ethylene cross talk through interaction of *ARF7* and *ACS5*. In this model, both *ACS5* and *ARF7* promoters contain an ERE site (Table 4), suggesting that they are both stimulated by ethylene. In addition, the promoter of *ACS5* gene has a site for ERF1 (GCC box), indicating strong ethylene feedback regulation (Table 3). The *ACS5* promoter also contains an AuxRE site which interacts with ARF dimers.

Auxin-responsive genes which contain an AuxRE site are inhibited by the binding of Aux/IAA repressor proteins with ARF in the absence of auxin. In the presence of auxin, an interaction between a TIR1 protein and Aux/IAA repressor proteins causes the degradation of Aux/IAA repressor proteins, resulting in the release of transcription factor ARF7 to dimerize and become active. In the proposed model, the ARF7 dimer can then bind to the AuxRE site in the promoter region of *ACS5* thus stimulating transcription of that gene. An Increased level of the ACS5 enzyme stimulates the production of ethylene in the plants. Increased ethylene binds to the ethylene receptor and signal transduction leads to the activation of the transcription factor EIN3. EREs in *ARF7* and *ACS5* promoter regions are target sites for EIN3 binding, which can then serve as a stimulus to both genes. EIN3 binding to the ERE site of the *ARF7* promoter region will activate

the transcription of *ARF7* and release its protein ARF7 to be used to bind to an auxin response element, further regulating them. The *arf7* mutant results in a disruption of cross talk at the transcriptional regulation of *ACS5*. Therefore, the cross developed by this project, allowed the evaluation of *ACS5* expression with normal ARF7 activity and without the ARF7 transcription factor.

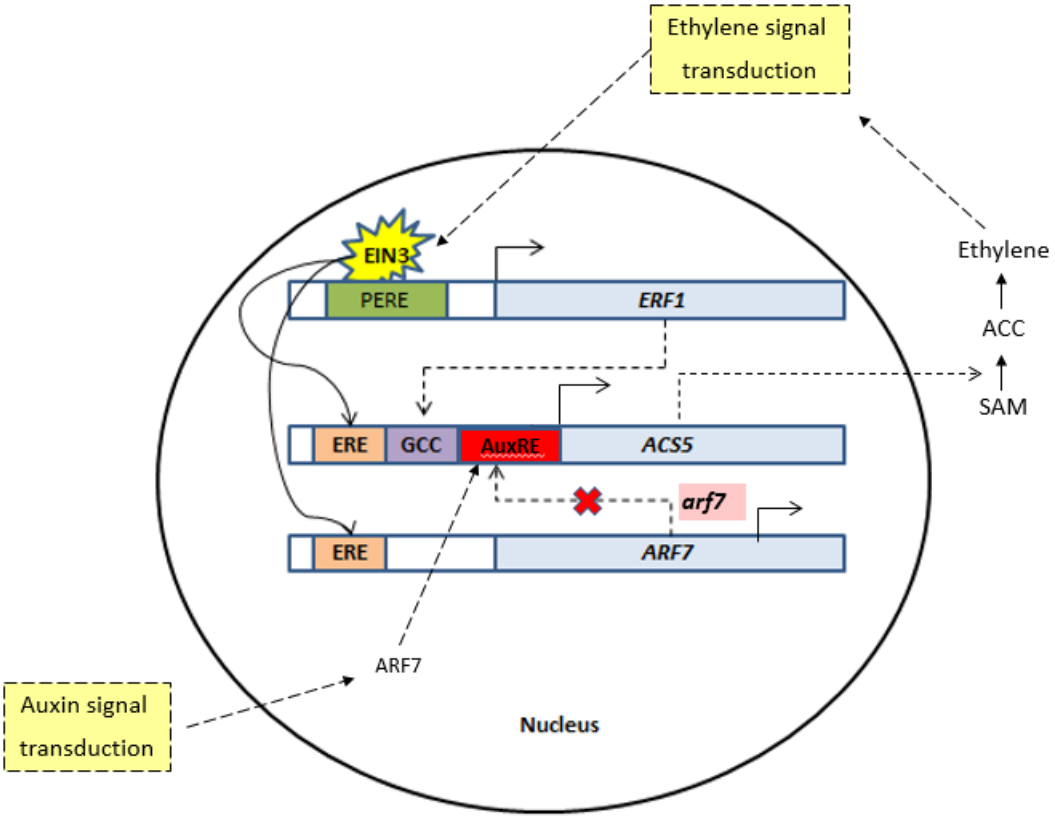


Figure 19. Cross talk model of interaction of auxin and ethylene at the transcriptional level.

The disruption of auxin/ethylene cross talk in the *arf7* mutant, was demonstrated using the *GUS* reporter system allowing the visualization of *ACS5* expression in plant tissues. The *ACS5p::GUS* construct was originally developed by Tsuchisaka and Theologis who demonstrated the expression of *ACS5* in the hypocotyl tissue of dark-grown seedlings, after wounding, and after treatment with exogenous application of IAA

(Tsuchisaka and Theologis, 2004a). In the reported experiments, the *ACS5* expression was observed in the area next to the 2.5 μM or 5.0 μM IAA agar blocks in the *ACS5p::GUS* seedlings (Figure 13). Highest *ACS5* expression was observed in the hypocotyls of *ACS5p::GUS* seedlings with 5.0 μM IAA treatment. However, no *ACS5* expression was observed adjacent to the IAA agar blocks in hypocotyls of the *ACS5p:: GUS x arf7* cross seedlings, although there was clear expression in the apical meristems and cotyledons of those plants. Therefore, it is interpreted that this lack of expression is due to the lack of ARF function in the *ACS5p::GUS x arf7* cross which diminished transcription of *ACS5*.

Further evidence of cross talk was provided through ethylene measurements. Stem and leaf tissues from *ACS5p::GUS* and wild type plants showed a greater amount of ethylene production than that of the *arf7* mutant (Figure 18). The lower production of ethylene in the *arf7* mutant may involve the lack of cross talk between ARF7 dimers and the *ACS5* gene. The parent gene *ARF7* produces a viable protein to activate transcription in *ACS5*; hence ethylene production levels are higher in *ACS5p::GUS* than it is in *arf7*. This suggests that without ARF7, *ACS5* is transcribed less resulting in lower ethylene biosynthesis. Also, the bioinformatical data indicate that promoters of *ACS4, ACS6, ACS8,* and *ACS11* also contain AuxRE response elements and likely respond to ARF7 activation as well. Interestingly, *ACS4, ACS8,* and *ACS11* are from the same subgroup as *ACS5* and can form active heterodimers with each other under different conditions (Tsuchisaka and Theologis, 2004b). Therefore, the observed lower ethylene production could be the result of lower transcription of *ACS5* along with other *ACS* family members.

It was also noted that ethylene production in *ACS5p::GUS x arf7* cross showed a lesser amount of ethylene production than that of *ACS5p::GUS* but greater than the *arf7* mutant. While the attempt was made to select homozygous plants for *arf7*, it is possible that the cross is heterozygous so one allele produces a functional ARF7 protein. It is known that in *Arabidopsis* plants, the T-DNA insertion can be lost in successive generations (Krysan et al., 1999). In order to produce enough seeds for experimentation, seeds were collected from F_3 and F_4 generations. Thus, a resulting heterozygous plant could account for the partial reduction in ethylene production compared to the *arf7* mutant. On the other hand, wild type and *ACS5p::GUS* are homozygous for wild type *ARF* and produce roughly larger amounts of ethylene during the first incubation period.

In the cross talk model, increased ethylene production triggers a signal transduction cascade which activates ethylene-responsive genes. In the proposed model above, EIN3 activation and binding to the ERE site of the *ARF7* promoter region will activate the transcription of *ARF7* and release more ARF7 which will bind to an auxin response element. Since *ACS*5 has AuxRE response elements, then increased ARF7 production could likely increase *ACS5* transcription in the presence of auxin. However, in *arf7* mutant plants, EIN3 binding will not result in the production of functional ARF7 proteins. Therefore, increased ethylene production does interact with auxin signaling in *arf7* mutant plants. This interpretation is consistent with experimental evidence that the *arf7* mutant is ethylene resistant as well as auxin resistant (Li et al., 2006).

The AtcisDB is specific for *Arabidopsis* and shows an ethylene response factor binding site for ERF1 (GCC box in the model) for ARF7, and not the ERE found by PLACE (Table 4). Since ERF1 activates secondary ethylene responsive genes, this is another potential mechanism for ethylene cross talk. Other EREs are located in the promoter regions of ethylene-induced genes that are targets for EIN3 binding as well. Therefore, EIN3 directly and indirectly regulates numerous other genes in response to ethylene.

Tissue localization of *ACS5* expression

In order for auxin to signal messages to the cells, auxin must be transported into cells and interact with transcription factors in order to generate a cellular response. Auxin is naturally made in the shoot apical meristems and in young tissues. In stems, auxin moves down from the shoot apical meristem generating a concentration gradient where the highest concentrations are near the tip. This high concentration of auxin stimulates cellular growth, but can also stimulate ethylene production when auxin reaches a higher level. Therefore, patterns of *ACS* transcription should parallel locations of high auxin accumulation. In general, in these analyses the tissue localization of *ACS5* expression shows increased expression in the areas where auxin levels should also be higher.

In five-day-old dark-grown seedlings, *ACS5* expression in the parent *ACS5p::GUS* plants is strong in the areas associated with high auxin concentration, the tip, young leaves and vascular tissue throughout the hypocotyl (Figure 11). However, *ACS5* expression is more localized to the apical area of *ACS5p::GUS x arf7* shoots,

with little expression below the upper portion of the hypocotyls (Figure 11). This may indicate the lack of functional ARF7 proteins in the cross reduces the level of auxin-regulated *ACS5* in the dark-grown seedlings.

The expression patterns previously observed in light-grown compared to dark-grown plants are consistent with reports that *ACS* family members are expressed differently in response to light (Tsuchisaka and Theologis, 2004a). For example, little ACS5 expression was observed in the vascular tissue in the hypocotyls of light-grown plants, but was strong in the cotyledons and developing leaves and their vascular traces, which are also areas associated with higher levels of auxin (Figure 12). The increased auxin serves to increase cell expansion and vascular tissue growth in young leaves, thus increasing their surface area so that the young seedlings can quickly photosynthesize.

In light-grown seedlings, the *ACS5* expression pattern in *ACS5p::GUS* x *arf7* cross is similar to the *ACS5p*::*GUS* parent plants showing strong expression in the cotyledons and young leaves and even in the hypocotyl vascular tissue. Therefore, the expression of *ACS5* does not appear to be regulated to the same extent in light-grown plants as for dark-grown plants.

Localization of *ACS5* expression was reduced in floral tissues in the cross. Lack of or lower levels of *ACS5* expression in the flowers of the cross indicated developmental differences in regulation of *ACS5* in the cross compared to the parent plant. *ACS5* is known to be expressed in floral tissue (Table 1), which is confirmed with these experiments.

It was also noted that there were *ACS5* expression differences during flower development, where mature flowers showed stronger expression in the stamen (male portion) of the flower; the filament and the anther (Figure 14). These results are consistent with localization studies of *ACS* family gene members which show evidence that expression is seen in early flowering developing tissues of the pedicel, stamen filaments, and sepals (Tsuchisaka and Theologis, 2004a). The developing flowers in the cross did not show *ACS5* expression in the floral parts, but did exhibit strong wounding-induced *ACS5* expression, indicating that the *GUS* reporter was acting in a similar manner as found in the parent plants. Overall, *ACS5* expression in floral tissue seems to be regulated by ARF7.

Gravitropism. The initial model proposes an interaction between auxin and ethylene in the developmental response, gravitropism (Figure 5). A plant's normal gravitational field is vertical; its natural position to reach for its main source of energy, the sun. In vertically-oriented stems, auxin is distributed equally. However, when reoriented to a horizontal position, auxin is redistributed along the stem and accumulates on the lower portion of the stem, causing these cells to elongate pushing the stem upward (Figure 3). It is proposed that this change in orientation may drive an increase in ethylene production at the transcriptional level (Figure 5). In this study, evaluation of cross talk between ARF7 and *ACS5* in *Arabidopsis s*eedlings showed that auxin and ethylene may interact at the transcriptional level through cross talk and could potentially regulate a developmental response, gravitropism. When five-day-old dark-grown plants were reoriented from their vertical growing position, both *ACS5p::GUS* and *ACS5p::GUS x*

arf7 showed upward curvature (Figure 14), and it is assumed that this curvature is a result of auxin redistribution to the lower portion of the stem. *ACS*5 expression was slightly increased throughout the hypocotyl tissue but did not show a distinct increase in the lower flank of the stem in either *ACS5p::GUS* or *ACS5p::GUS x arf7* hypocotyls seven hours after reorientation (Figure 16). As noted previously, the cross has less expression in the hypocotyl of dark grown plants.

Wounding. An early wounding study showed that when dark-grown pea plants were mechanically wounded, ethylene production increased nearly four times within one hour (Saltveit and Dilley, 1978). In young *Arabidopsis* leaves, wounding treatment of *ACS5p::GUS* and *ACS5p::GUS x arf7* showed a wounding response in those cells surrounding the wound (Figure 17). Strong wounding responses were also noticed for both parent and cross at the cut sites in excised flowers, hypocotyls, and petioles of light-grown seedlings (Figure 17). Since the wounding response was equally strong in both parent and cross, then it was concluded that the cross talk between ARF7 and *ACS5* is not a major factor in regulating the wounding response.

Future studies

User databases and promoter analysis tools are improving. Additional bioinformatical analysis will be able to give a more in depth look into the proposed questions of this study. Additional analysis of the ERF1 short sequence in the promoter regions from the other two search engines could indicate that these elements are for secondary genes.

In addition, specific molecular experiments are needed to experimentally establish the role of the EREs and AuxREs in the regulation of *ACS* and *ARF* genes. Basically, these experiments could confirm other aspects of the model for *ACS5* and *ARF7* as well as for other *ACS* and *ARF* family members.

Conclusions

Based on the experimental evidence provided here, binding of the transcription factor ARF7 may be considered a positive regulator of *ACS5* expression. Accordingly, in the presence of auxin, ARF7 protein binds to an AuxRE in the promoter of *ACS5* and regulates its transcription and stimulates ethylene production. Increased ethylene production will cause a cascade of events to activate its primary ethylene response gene, *ERF1*, by binding to a primary response element (ERE) in its promoter region. The ERF1 protein product will bind to a GCC box along with EIN3 binding to an ethylene response element in the promoter region of *ACS5*, thus activating transcription of *ACS5* and therefore stimulating ethylene production. With increased ethylene, EIN3 can also bind to an ethylene response element in the promoter region of *ARF7*. Once *ARF7* is transcribed, its protein product, ARF7, can then bind to an AuxRE in the promoter region of *ACS5* and activate its transcription, further stimulating ethylene production

However, when *ARF7* lacks its function, its mutant form *arf7* will not be able to produce a viable protein product which is needed to activate *ACS5* transcription by binding to an AuxRE in its promoter region. Therefore, it is interpreted that this

interruption in communication interferes with the regulation of *ACS5* and ultimately ethylene production. .

This model of ARF-regulated *ACS5* expression is demonstrated in *ACS5* expression patterns which indicates regulation during development (floral tissues and dark-grown hypocotyl tissues) and in response to IAA treatment. *ACS5* expression patterns in light-grown plants are associated with different stages of growth and growth conditions. In the cross, *ACS5* expression is not seen in the flowers compared to that of *ACS5p::GUS* (Figure 14). As auxin is produced at high levels in tissues such as cotyledons and young leaves, cross talk through activation of *ACS5* expression can result from the higher auxin levels and ARF7 activation. In dark-grown plants, *ACS5* expression was lower in the hypocotyls of the cross compared to that of wild type when exposed to IAA (Figure 15). Here, the lack of *ARF7* function is disrupting the cross talk between ARF7 protein and *ACS5*; therefore less *ACS5* is transcribed in that area. Therefore, it is concluded that *ACS5* expression is regulated differentially in light-grown compared to dark-grown plants. In addition, cross talk was not observed during gravitropic curvature and after wounding, indicating other mechanisms of transcriptional regulation for these responses. In these cases, other regulatory mechanisms may come into play.

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APPENDIX A

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

May 6, 2015

Marcia Harrison-Pitaniello
Department of Biological Sciences
Marshall University
Huntington, WV 25755
Office: Science 200A

Dear Dr. Harrison-Pitaniello:

This letter is in response to the submitted thesis abstract for Mia Lynne Brown entitled *"Evaluating Potential Plant Hormone Cross Talk between Auxin and Ethylene in Arabidopsis."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

CURRICULUM VITAE

Mia Brown

Education

M.S. Biological Science, Marshall University, 2015

Business Management Foundations Graduate Certificate, Marshall University, 2009

M.S. Adult and Technical Education, Marshall University, 2009

B.S. Biotechnology Research, Marshall University, 2008

Employment

Professional Development in Education

- **Adjunct Faculty Allied Health and Sciences**
  Mountwest Community & Technical College, 2011-current
  - **Biology 101L**- Integrate basic biological principles with the effects of these principles in the everyday life of man through lecture presentations.
  - **Biology 101**- Integrate basic biological principles with the effects of these principles in the everyday life of man through laboratory experiments.

- **Adjunct Faculty General College Studies**
  Mountwest Community &Technical College, 2011-2012
  - **College Success 101**- Provide freshman and new transfer students with an opportunity to adjust to the academic and social environment of college under the guidance of a faculty/staff mentor.

- **Academic Skills Instructor**
  Mountwest Community & Technical College, 2009-current
  - Tutor students in adult and basic education skills such as math and study skills

- **H.E.L.P (Higher Education Learning Problems) Graduate Assistant,** Marshall University, 2008-2010
  - Tutored students who have specific challenges in learning due to a specific Learning Disability and/or Attention Deficit Disorder/Attention Deficit Hyperactivity Disorder.

Professional Development in Business Management
District Leader/Manager
Deb Shops, June 2014 to April, 2015

- Promoted to oversee multi-units in the West Virginia market following superior-rated performance as store manager in the #1-rebranded store in the company. Managed an $8M area.
- Implemented new marketing and branding strategies for the West Virginia Clothing Voucher program that generated a 1.04 multiplier during the company's 5 week back-to-school peak season.
- Actively controlled shrink and successfully removed stores from high concern list.
- Developed recruitment strategies and training programs to create an inviting selling culture throughout the area.
- Successfully developed a customer focused selling strategy, "3 +1", to ensure company key performance indicator expectations were exceeded; district ranked #1 in average dollar sale (ADS) within the company.

- Coached and developed team members through sell down strategies centering on GOB sale.

Store Manager
Deb Shops, March 2012 to June 2014
- Represented the region in developing companywide visual concepts to support newest fashion trends.
- Served as an onboarding district trainer for the region.
- Successfully rebranded a low performing store and achieved +20% COMP and repositioning to Top 10 within the company

Assistant Store Manager, Interim Store Manager
Hollister 2010-2012
- Managed a staff of 75 part-time employees, 3 full-time assistant managers, and 1 full-time stock coordinator.
- Consistently recruited, hired, and developed those who represented the brand in totality.
- Upheld company standards and protocol to run an effective business that produced superior results.
- Oversaw all aspects of management for a high volume store.
- Provided a work environment that was fair, developmental, and focused on customer service.

Assistant Store Manager
wet seal, 2007-2008

Associate Store Manager
Charlotte Russe, 2006-2007
- Oversaw all aspects of management in the absence of the store manager.
- Helped manage all part-time employees.
- Active team member in meeting and surpassing monthly sales goals.

Sales Lead
EXPRESS, 2005-2006
- Scheduled entry-level sales associates.
- Trained new workers to ensure employees used proper protocol at all times.
- Led sales segments throughout the day with a competitive attitude.

Professional Development in Scientific Research
- **Graduate Student Thesis:** *"Potential Hormonal Cross-Talk Between Auxin and Ethylene",* Marshall University, 2012-2015
- **Undergraduate Research Assistant,** Marshall University, Huntington, WV (2004-2008)
- **Lab Technologist GS-4**, AFRS-USDA, Kearneysville, WV (2003-2004)
- **Biological Science Aid GS-1**, AFRS-USDA, Kearneysville, WV (2000-2003)

Honors/Certificates
- **Outstanding Black College Student Honor Society Alumnae,** Marshall University, 2003-2008
- **NASA Scholar,** Marshall University, 2004-2007
- **West Virginia Sigma Xi Awardee,** Marshall University, 2004-2007
- **West Virginia Academy of Science Awardee,** Marshall University, 2003-2007