

A Physiological and Evolutionary Study of the Plant Hormone Ethylene

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by

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ABSTRACT

The plant hormone ethylene plays many roles in the development and physiology of plants, such as coordination of ripening, wound response, and cell fate specification. The final step of the ethylene biosynthetic pathway is catalyzed by 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase or ACO). There are five ACO genes in *Arabidopsis thaliana*, all coding for different isozymes of ACO. The expression domains in the plant and expression levels of all five ACO genes are not well understood. Part of the aim of this thesis is to elucidate the expression domains and levels of the five ACO genes in *Arabidopsis* using promoter::GUS fusions and quantitative reverse transcription PCR (qRT-PCR) analysis, respectively.

The genus *Selaginella* is part of the lycophyte family of plants. *Selaginella* species are heterosporous, vascularized, microphyllous plants. The processes of ethylene perception and biosynthesis are poorly understood in *Selaginella*. The second part of this thesis will focus on the study of ethylene perception in *Selaginella apoda* and *Selaginella moellendorffii*. Both *S. apoda* and *S. moellendorffii* were exposed to exogenous ethylene for 60 days, after which leaf cell size measurements were made using the scanning electron microscope. Sporangium counts were also performed, looking for sex and positional data on the strobili. In ethylene treated plants, cell sizes in both plants were shown to be significantly smaller. Also, the ratio of megasporangia to microsporangia produced by strobili on *S. apoda* plants increases dramatically when the plants are grown in ethylene exposure conditions.

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INTRODUCTION:

OVERVIEW

Hormones are physiologically active substances that are produced in one part of an organism but have effects in remote regions of the organism. Plant development, stress response, cell fate, and reproductive activities are all guided by the five canonical plant hormones. One of these, ethylene, is the simplest alkene, and a gas, which is unique for plant hormones (*Figure 1*). In spite of this chemical simplicity, ethylene is an important component in signaling pathways that guide developmental, physiological, and stress responses in plants. As ethylene has such a diverse range of effects, it is of tremendous importance to humans, particularly in agronomics, where its effects on the senescence, ripening, and spoilage of fruits and vegetables affect their shelf-life. In spite of this great importance, and a long history of research, there is still much to be learned about ethylene.

Ethylene was discovered as being physiologically active in plants by Dimitry Neljubov in 1901. Observing that illuminating gas was causing trees to lose their leaves

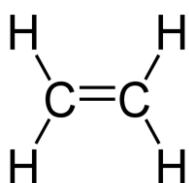


Figure 1: A 2D representation of the structure of ethylene

more quickly than normal, Neljubov tested the constituents of illuminating gas and found that ethylene was the component that caused the observed senescence. Later, in the 1930's, a group of scientists from the Boyce Thompson Institute published a series of papers which described many of the physiological and

developmental effects of ethylene (Abeles 1973). Also, in 1934, it was shown that plants produce ethylene, and it began to be called a plant hormone. This nomenclature has always been interesting because ethylene is a gas, and plants have no way of regulating its movement or transport, as is the case with other plant hormones. Thus, when ethylene

is produced, it is likely to have widespread effects. This research aims to not only elucidate patterns of expression of ethylene biosynthesis genes in flowering plants, but to also explore evolutionary links between the ethylene biosynthesis pathways in flowering plants and more highly diverged, or basal, plants.

ETHYLENE IN PLANT DEVELOPMENT AND PHYSIOLOGY

Angiosperm Flowering and Sex Determination

Ethylene is understood to play a vital role in flowering and reproduction in angiosperms, or flowering plants. For example, in bromeliads, such as pineapple, ethylene is used to induce flowering (Lin et al. 2009). Ethylene is also linked to flower abscission and senescence. Ethylene is also a significant regulator of ripening in such fruits as tomato, avocado, and banana. These fruits are deemed “climacteric” fruits, and the burst of ethylene production at the outset of the ripening cycle is also accompanied by increased levels of respiration (Lin et al. 2009). Ethylene serves the purpose of coordinating ripening events in a fruit that would otherwise not be able to be synchronized (Giovannoni, 2004). The role that ethylene plays in many crop fruits affects humans and is very valuable economically, thus meriting further study.

Cell Growth and Cell Fate

Ethylene also has effects on cell growth and cell fate. Ethylene is a negative regulator of cell size (Keiber et al. 1993). It does, however, positively regulate root hair growth (Tainmoto et al. 1995). In the Tainmoto et al. paper, a model is proposed that ethylene produced in the endodermis moves through the apoplastic space between cortical root cells, and when it finally reaches an epidermal cell through the apoplastic

space, that epidermal cell will grow a root hair as a response to the ethylene signal (*Figure 2*). This happens because, as a result of the ethylene travel through the apoplastic space, root epidermal cells produce differentially robust ethylene responses. The cells that have a high enough level of response will produce root hairs (Tainmoto et al. 1995).

Ethylene has an effect on roots through the root-nodulation process in legumes (Wang et al. 2002). Nodules form when *Rhizobium* fungi infect roots, forming a symbiosis between the plant and the fungus. Heidstra et al. (1997) showed that root nodule primordia are given positional information by the plant, guiding where the nodules eventually form. This is achieved by ethylene production in portions of the root, inhibiting formation of nodules. Thus, nodules are left to form where the ethylene is not produced (Heidstra et al. 1997). In the hyper-nodulation mutant *sickle (skl)*, in which many more nodules form than usual, ethylene insensitive phenotypes also present themselves in the form of delayed leaf and seedpod abscission, and delayed petal senescence. It has also been shown that for a nodule to actually form, ethylene production must be stopped, as ethylene has an inhibitory effect on nodule formation (Wang et al. 2002).

Wound Response

The wound response in plants is also mediated by ethylene. In tomatoes, ethylene and Jasmonic acid work together to upregulate wound response genes (O'Donnell, et al. 1996). Wounded mung bean seedlings produce up to 40 fold greater levels of ethylene than in the unwounded state (Yu and Yang, 1980). There is a similar response in tubers-

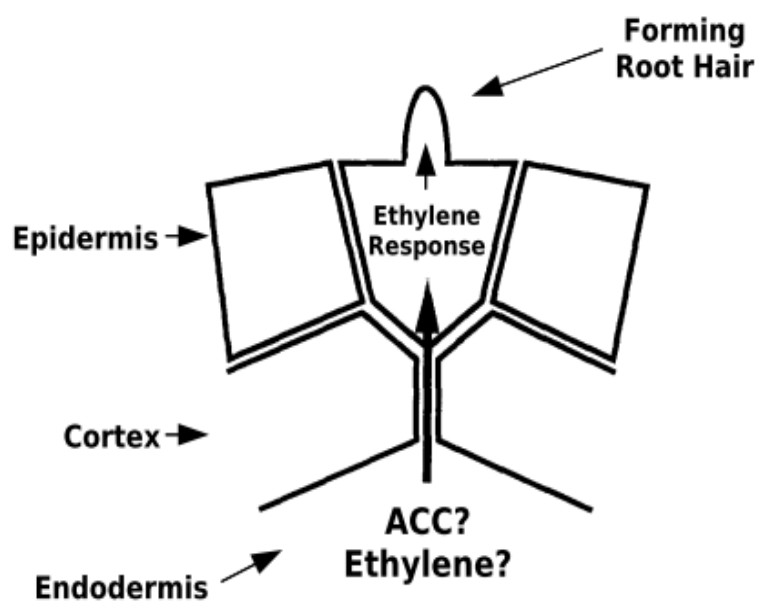


Figure 2: Ethylene has effects on root hair specification. Ethylene produced in the endodermis travels through the apoplastic space between cortical cells and induces ethylene responses in the epidermal cells it reaches, which in turn induce root hair growth (Modified from Tainmoto, et al. 1995).

when they are wounded, ethylene production increases dramatically (Lulai and Suttle, 2004). In tobacco, ethylene plays a role in the response to insect wounding. When caterpillars feed on the leaves of the tobacco plant, their oral secretions elicit an ethylene burst from the plant which coordinates the response (Kessler and Baldwin 2002). It was also shown in maize that when ethylene synthesis was inhibited, more leafy tissue was eaten by caterpillars, thus ethylene coordinates defense responses (Harfouche et al. 2006). Ethylene is clearly a part of the wound response of many types of plants, highlighting the importance of more research in this field.

ETHYLENE SIGNAL TRANSDUCTION

Most of what is known about ethylene signal transduction comes from molecular genetics experiments with *Arabidopsis thaliana*. *Arabidopsis* is an important model organism in plant biology. It is useful in this capacity because it has a sequenced genome, its generation time is comparatively short, it is easily transformable, and the plant itself is physically small, which facilitates the growth and storage of large numbers of plants in sometimes limited lab space.

The ethylene signal transduction pathway (*Figure 3*) is complicated and far from completely elucidated. Ethylene perception is mediated by a family of five Golgi and ER-membrane bound receptors ETR1 (ETHYLENE RESPONSE 1), ETR2, ERS1 (ETHYLENE SENSOR 1), ERS2, and EIN4 (ETHYLENE INSENSITIVE 4). All of these receptors bind ethylene in their N-terminal domain and are all related to each other with a range of 57%-79% sequence identity to one another (Hua and Meyerowitz, 1998). These receptors were discovered as dominant gain of function mutants—when their gain of

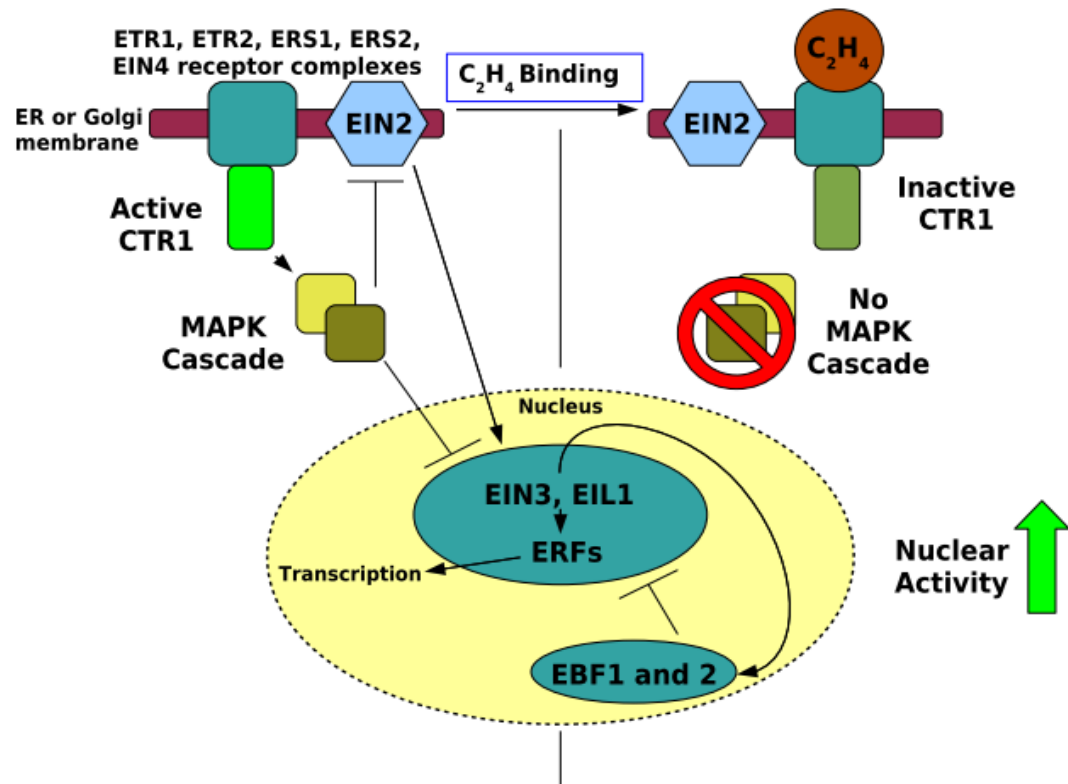


Figure 3: The ethylene signal transduction pathway. ETR1, ETR2, ERS1, ERS2, and EIN4 are the ethylene receptors, to which the constitutively signaling CTR1 is coupled. When CTR1 is active, it putatively phosphorylates a MAPK cascade, which downregulates EIN3 and EIL1, ethylene response transcription factors. When ethylene binds one of the receptors, CTR1 is deactivated and the MAPK cascade does not get phosphorylated. Because of this, EIN3 and EIL1 are allowed to build up in the nucleus, expressing ethylene response factors and inducing transcription. EIN3 and EIL1 self-regulate by upregulating EBF1 and EBF2, which suppress EIN3 and EIL1 (Modified from Yoo, et al. 2009).

function phenotype is expressed, they do not perceive ethylene and constitutively signal. Quadruple loss of function mutants for *etr1*, *etr2*, *ein4*, and *ers2* in *Arabidopsis* show constitutive ethylene responses, which implies that instead of activating a pathway, these receptors negatively regulate ethylene responses when there is no ethylene present (Hua and Meyerowitz, 1998).

The ethylene receptors inactivate CTR1 (CONSTITUTIVE-TRIPLE-RESPONSE-1), which resembles a Raf-like protein kinase (Yoo et al. 2009). It has been shown in animals that Raf-like protein kinases activate mitogen-activated protein kinase (MAPK) phosphorylation cascades (Pelech and Sanghere, 1992) and that CTR1 shares this function. CTR1 has also been shown to bind to the receptor complex (Yoo, et al, 2009; Hua and Myerowitz 1998; and Clark et al. 1998). It is also a negative regulator of ethylene responses, as shown in loss of function mutant studies (Keiber et al. 1993). When CTR1 is active, it putatively phosphorylates downstream MAPK kinases (MAPKKs), which in turn activate MAPK target proteins (Wurgler-Murphy and Saito, 1997). In the absence of ethylene, CTR1 is constitutively signaling, which leads to the downregulation of downstream transcription factors that induce ethylene responses (Yoo, et al. 2009, and Wurgler-Murphy and Saito 1997). However, when ethylene is present and binds to the receptors, CTR1 is inactivated, which allows the positive regulation of ethylene response transcription factor gene expression. Also, when ethylene is present, levels of transcripts of ERS1, ERS2, and ETR2 increase, as well as transcripts for the protein RTE1 (REVERSION TO ETHYLENE SENSITIVITY 1). The increase in levels of RTE1 transcripts is interesting considering that RTE1 is a suppressor of ethylene signaling. It has been shown that overexpression of RTE1 actually lowers the sensitivity

of a plant to ethylene (Resnick, et al. 2006 and Yoo, et al. 2009). This may be a means of keeping ethylene responses from becoming too strong and damaging the plant, as well as a way to fine-tune the response of the pathway by adding another regulation point for the ethylene response.

EIN2, which is also an ER membrane bound protein, has been shown in *Arabidopsis* to confer complete ethylene insensitivity on a plant in loss-of-function mutants (Alonso et al. 1999). It is also a regulator of the ethylene transcription factors, as it is thought to be an intercessor between CTR1 and the ethylene response transcription factors. Expression of only the C-terminal end of the EIN2 protein in *ein2* null mutants was enough to restore ethylene responses where there previously were none (Alonso et al, 1999). EIN2 has been shown to be related to the Nramp family of metal transport proteins. This could be related to ethylene signaling because copper is required by ethylene receptors to bind ethylene. The membrane localization of EIN2 is suggestive of a relationship between it and the ethylene receptors, but its specific function is still the topic of scrutiny and there is still much more work to be done in order to elucidate the function of EIN2.

The ethylene response transcription factors, EIN3 and EIL1 accumulate in the nucleus in response to ethylene presence. EIN3 is the main ethylene response transcription factor, and it is constantly being degraded at low levels by the 26S proteasome. While EIN3 enhances expression of genes that carry out the ethylene response, it also enhances expression of genes that produce proteins which negatively regulate EIN3. These proteins are EBF1 and EBF2 (EIN3 binding F-box proteins 1 and 2). It has been shown in *Arabidopsis* that in *ebf1 ebf2* null mutants, EIN3 accumulates at

higher levels than normal, which eventually causes seedling lethality because of the inhibitory effects of the ethylene response. Thus, by enhancing the expression of its own negative regulators, EIN3 levels are kept from accumulating too much and harming the plant. It has also been shown that EBF1 and EBF2 have different roles in regulating the ethylene response. EBF1 acts during the beginning stages of ethylene signaling, while it seems that EBF2 acts in the later stages of ethylene signaling (Yoo, et al. 2009).

ETHYLENE BIOSYNTHESIS

In angiosperms, ethylene biosynthesis is achieved by a three step process (*Figure 4*), and begins from a common biosynthetic precursor, methionine. Methionine is converted to S-adenosyl-L-methionine (S-AdoMet) by S-AdoMet Synthetase (SAM Synthetase). S-AdoMet is also common in higher plants, frequently acting as a methyl donor (Wang et al. 2002). The first committed step in ethylene synthesis happens when S-AdoMet is converted to 1-aminocyclopropane-carboxylic acid (ACC) by ACC synthase (ACS). ACC is then converted to ethylene and 5'-methyladenosine (MTA) by ACC oxidase (ACO) (Lin, et al, 2009). In higher plants, valuable methionine is regenerated by what is now called the Yang cycle (reviewed in Yang and Hoffman 1984). The cycle is a way of using the MTA left over from the ACC synthesis reaction and recycling it, turning it back into methionine. First, MTA is degraded into methylthioribose (MTR) and adenine, followed by a phosphorylation of MTR. The MTR is then oxidized into α -Keto- γ -methylthio-butyric acid (KMB), which then has an amino group added to form methionine (Yang and Hoffman 1984).

The creation of ACC catalyzed by ACS is thought to be the most common rate-

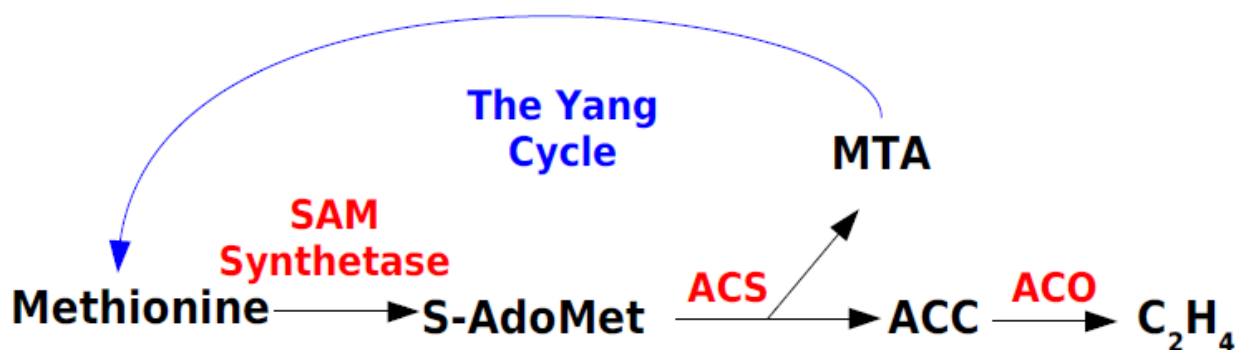


Figure 4: The ethylene biosynthesis pathway. Methionine is converted to S-ado-Met by SAM synthetase. Then, SAM is converted to ACC by ACC synthase. The Yang cycle recycles the leftover molecules from this reaction and regenerates methionine from them. The final step in the ethylene biosynthesis pathway, the formation of ethylene from ACC, is catalyzed by ACC oxidase.

limiting step of the ethylene biosynthetic pathway. Evidence for this hypothesis is that when ACC is added to a plant system, the levels of ethylene produced increase in a one-to-one fashion (Osbourne 1996). It has been shown that ACC oxidases in tomato are very highly regulated and sensitive to stimuli such as mechanical wounding and ripening of tomato fruit (Barry et al. 1996). It is also possible that instead of ethylene transport through the plant, that ACC is synthesized elsewhere in the plant and then transported in aqueous form to destination cells, at which time the ACC is converted to ethylene by ACO (Tainmoto et al. 1995). This shows that ACC oxidase may be a regulator of ethylene biosynthesis. If ACO genes are highly regulated and have highly tissue-specific expression, their expression can be finely tuned to produce as much ethylene as is needed, giving ethylene another regulation point past its rate limiting step.

ACC SYNTHASE

1-aminocyclopropane-1-carboxylate synthase (ACC synthase or ACS) is the

enzyme that catalyzes the rate limiting step in ethylene formation (Tsuchisaka and Theologis, 2004). It is encoded for by a multigene family, whose twelve amino acid sequences show identity from 34% to 91% (Yamagami et al. 2003). ACS proteins are dimeric and belong to the type I pyridoxal 5' phosphate (PLP) -dependent enzyme family, which uses PLP as a cofactor in its action (Capitani, et al. 2005). In a 30,000:1 ratio, ACS preferentially catalyzes two reactions: the most abundant is the conversion of S-AdoMet to ACC, and the second is the conversion of S-AdoMet to vinylglycine, which irreversibly inhibits ACS activity, rendering the enzyme useless (Kende 1993).

Given the very high number of isozymes for ACS, it is important to elucidate the reason for the large number and the function of the different genes. Is there some sort of functional redundancy? Or is there none at all and each gene has a separate function? It has been shown that many ACS genes are highly activated in many parts of the plant, frequently in overlapping patterns, suggesting physiological specificity but also some degree of functional redundancy (Tsuchisaka and Theologis, 2006). Based on GUS transcriptional reporter fusion experiments in *Arabidopsis thaliana*, it is known that many of the ACS enzymes, most notably ACS2 and ACS8, are expressed at high levels ubiquitously in the plant. These experiments have also shown that some ACS genes, such as ACS9 and ACS11, are not expressed very highly, but show a dynamic expression in few tissue types throughout development (Tsuchisaka and Theologis, 2006). This provides evidence for both functional redundancy and specialization of function within this gene family.

ACC OXIDASE

ACO is a member of the 2-oxoglutarate dioxygenase family of enzymes. The dioxygenases are enzymes that incorporate oxygen from O₂ into some substrate. Frequently in these reactions, iron (Fe) is a cofactor, but this is not always the case (Prescott and John 1996). Also, some of the members of this family must have 2-oxoglutarate present for activity while others, such as ACO, do not require 2-oxoglutarate and utilize another co-substrate, such as ascorbate (Prescott and John 1996).

ACO is an approximately 35 kDa tetramer (Dong, et al. 1992). ACC binding to the active site is mediated by iron (Zhang et al. 2004). The next step is O₂ fission, which completes the oxidation reaction and creates an ACO-Fe (III) species. This product is then reduced by ascorbate to ACO-Fe (II), now ready to catalyze another ACC oxidation reaction. It is also thought that ascorbate may also bind the enzyme, helping to create a more favorable enzyme-substrate complex through allosteric activation, by which it helps the reaction proceed in a manner outside of its reductive capabilities (Zhang et al. 2004).

A main regulator of ACO activity is carbon dioxide (CO₂). It was shown that ACO activity is affected by increases in CO₂ concentration (Dong et al 1992). This was demonstrated with ACO proteins from apple, where ethylene production rises sharply with CO₂ concentration until about 4% CO₂ in the air, where the increase plateaus (Dong et al. 1992). Dong et al. also showed that if no CO₂ is present, ACO activity is completely stopped. It is thought that undissociated CO₂ is the active species that increases ACO activity. While it is converted to bicarbonate *in vivo*, the activity of ACO stays fairly constant over a wide range of pH while the CO₂ levels stayed the same. This suggests that even if the bicarbonate levels vary wildly, the ACO activity is still dependent on the

level of CO₂ in the system (John, 1997). The question is still up in the air about whether levels of CO₂ are a regulatory mechanism *in vivo*, considering that the levels described are mostly from *in vitro* studies and it is not known whether CO₂ reaches such high levels *in vivo*.

There are five ACO genes in *Arabidopsis thaliana*. It is probable that the isozymes of ACO, similar to the isozymes of ACS, are evolved for different physiological niches in the plant, and possibly functional redundancy. Patterns such as this are seen in ACS, but to what extent does ACO gene expression overlap and to what extent is it physiologically distinct? Preliminary microarray data from Genevestigator (Hruz et al. 2008), which is a database of microarray and gene expression data for model organisms, suggests that ACO2 and ACO4 are highly expressed throughout development. Also, the data show that several parts of the plant, such as the rosette leaves and hypocotyls, co-express these two genes in high amounts. The Genevestigator data also provide preliminary evidence for physiological specialization, as ACO genes are differentially expressed in all parts of the plant. However, while these Genevestigator data are a suitable starting point for forming hypotheses, microarray data do not provide sufficient anatomical resolution, and are not exact enough to be regarded as an end point for gene expression questions. Experiments such as GUS transcriptional reporter fusions and quantitative reverse transcription polymerase chain reaction (qRT-PCR) are necessary to confirm the microarray data, but they will also provide better spatial resolution and more exact gene expression data than microarrays.

Studies like the Tsuchisaka and Theologis (2004) experiment discussed in the ACS section have not been performed for ACO genes. From those experiments, some

ACS genes seem to be expressed much more overall than others, which implies that they may be more important. Also, for the genes that are expressed at low levels, are those genes less important, or is their function much more specific than that of the broadly expressed genes? This thesis research sought to provide more concrete data and answer these and other questions for ACOs. First, where are the genes physically expressed in the plant? Are there certain genes that are specific to the leaves, the flowers, or any other part of the plant? Also, if one of these genes is knocked out, is there an upregulation of another ACO gene to fill in the enzyme activity gap left by the knocked out gene? Answering these questions about ACO is a boon to plant research, especially in the agricultural field. Also, Ashley Decarme of the Engstrom lab is working on a cross-species complementation of *Arabidopsis* ACO genes into *Selaginella moellendorffii*, a basal plant. Functional redundancy and expression data are important to help her to realize her goals.

EVOLUTION AND SEX DETERMINATION

The genus *Selaginella* is a part of the monophyletic lycophyte group. Lycophytes are among the earliest land plants (*Figure 5*), and were most abundant during the Devonian to mid-carboniferous period. There are only three extant groups: the Lycopodiaceae, Isoeteaceae, and Selaginellaceae. Selaginellaceae are heterosporous, which means that they produce two types of spores: megaspores, which are contained in the “female” megasporangia, and microspores, which are contained in the “male” megasporangia (*Figure 6b*) (Banks 2009). The spore producing structure is called a strobilus (pl. strobili) (*Figure 6a*). Genome size for *Selaginella* species is variable.

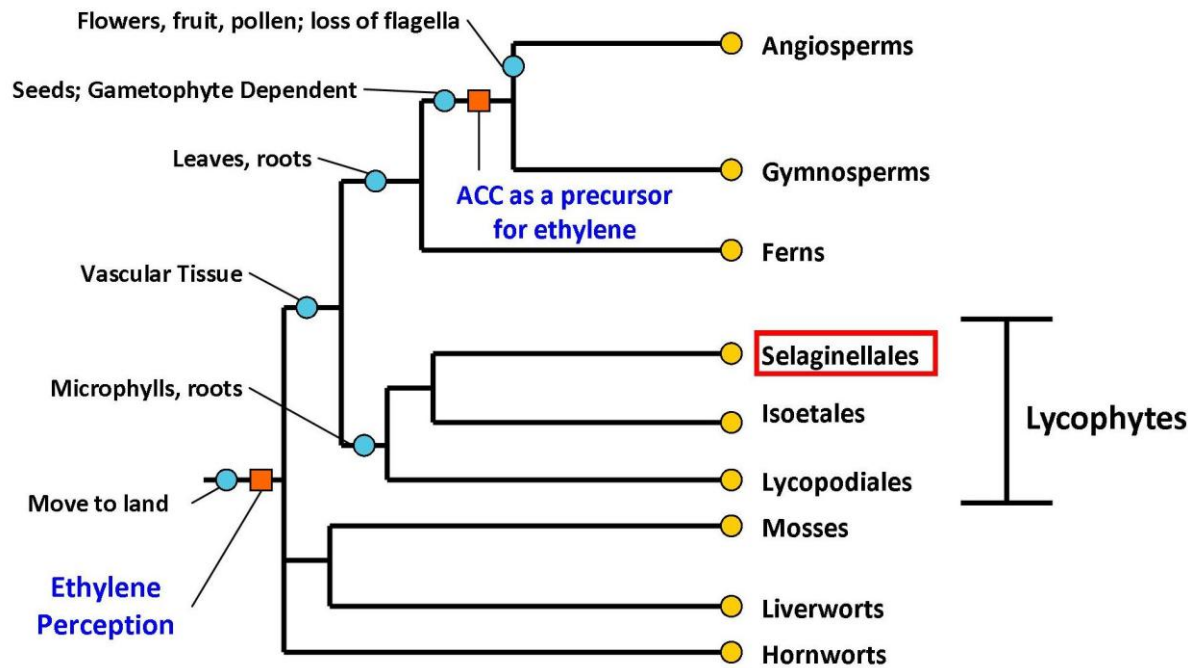


Figure 5: Phylogenetic Tree of Plants. The Lycophytes are indicated by the vertical arrow at right, and the Selaginellales is enclosed by the red box. The Selaginellales are distinguished from plants below them by the evolution of vasculature, roots, and microphylls, which are simple leaves that are small and only have one vein running through their center (adapted from Banks, 2009).

Selaginella moellendorffii has a genome size of ~110 Mbp, which is one of the smallest plant genome sizes that has been reported (Banks, 2009). For comparison, *Solanum lycopersicum* (tomato) has a genome size of ~950 Mbp (solgenomics.net). The small genome size and evolutionarily basal status of *Selaginella* species has led *Selaginella moellendorffii* to be selected as a model genetic organism.

Ethylene biosynthesis and perception are two open questions in *Selaginella* biology. The conventional wisdom is that all land plants produce ethylene (Osborne et al.

1996); however, as of yet, *Selaginella*

moellendorffii and *Selaginella apoda* have not had their ethylene production measured. There is evidence for production of ethylene in other basal plants, such as Liverworts, Mosses, and Ferns (Osborne et al. 1996); however, this skips over the Lycopod family completely.

The Osborne et al. (1996) paper also provides evidence for a non-ACC consuming ethylene biosynthesis pathway in basal plants. When

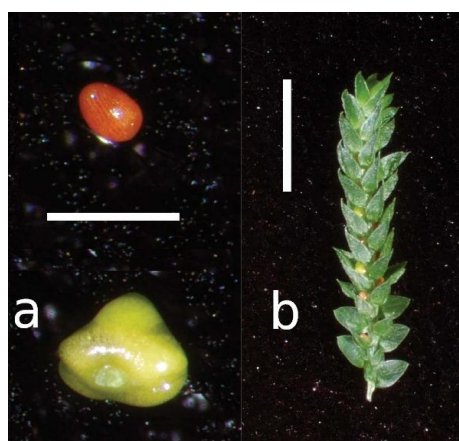


Figure 6: a. Microsporangium (top) and megasporangium (bottom). Scale bar is 1 mm. b. Strobilus from *Selaginella apoda*. The sporangia are visible in the lower portion as the ligules are open there. Scale bar is .5 cm.

ACC was fed to a broad selection of higher and basal plants, the higher plants all had increases in ethylene production, while the basal plants did not. Next, radioactive ^{14}C -containing ACC was fed to a smaller selection of of the same higher and basal plants, and the higher plants produced ethylene that contained the labeled carbon, while the lower plant phyla all produced ethylene, but it did not contain any of the radioactively labeled carbon. This suggests that lower plants, while producing ethylene, do not use ACC to

make that ethylene. It has also been shown that *Selaginella* species perceive ethylene. In a 1973 paper, Kenton Brooks explored the effects on *Selaginella* species in terms of phenotypic changes when ethephon, an aqueous ethylene-releasing compound, was applied to *Selaginella* plants. What he found was that the plants that had ethephon applied to them produced almost exclusively megasporangia, while the control plants produced mostly microsporangia. The question of perception is also open. This study is partly designed to address this question, but it is not known whether the perception machinery in *Selaginella* is the same as it is in higher plants. A BLASTp search reveals that *Selaginella moellendorffii* has putative versions of ethylene perception and biosynthesis genes (Table 1), but it has not been ascertained yet whether the putative ACOs found in *Selaginella moellendorffii* are functioning as ACOs or as some other enzyme. This is the reason for the cross species complementation assays that were

Table 1: BLASTp results for Arabidopsis ethylene biosynthesis and perception genes in *S. moellendorffii*.

<i>Arabidopsis</i> ethylene perception/ biosynthesis gene	CTR1	ACS1	ETR1	EIN3
<i>Selaginella</i> <i>moellendorffii</i> homolog? Name?	Yes: gw1.87.76.1	Yes: e_gw1.0.2222.1	Yes: e_gw1.40.82.1	Yes: e_gw1.43.121.1

discussed above.

There is precedent for independently evolved pathways in vascular plants such as *Selaginella*. It has been shown that syringil lignin, a main component in plant cell walls, is synthesized by an independently evolved pathway in basal plants (Weng et al. 2008). Based on the evidence offered by Osbourne (1996), it is entirely possible that the ethylene biosynthetic pathway in *Selaginella* could have evolved independently from the

pathway in higher plants. An independent pathway could be agronomically important in terms of crop engineering. Since a crop such as tomato has never expressed a *Selaginella* ethylene creation enzyme, it might not be able to regulate the enzyme as well, which would allow scientists a much finer level of control of the ethylene production process, since endogenous enzymes that are normally targeted by ubiquitination or are members of tightly regulated pathways are much harder to regulate through engineering.

This thesis has disparate aims, all tied together by ethylene. First, do *Selaginella apoda* and *Selaginella moellendorffii* perceive ethylene? Does ethylene application induce greater numbers of megasporangia in *S. apoda*? What are the expression patterns of the ACO genes in *Arabidopsis*? To what extent do the ACO genes in *Arabidopsis* share function, and to what extent are they distinct in function? Are the *Arabidopsis* null ACO lines complete knockouts? Finally, what are the levels of ACO gene expression in *Arabidopsis*?

MATERIALS AND METHODS

SELAGINELLA ETHYLENE ASSAYS

Perfusion Chamber Setup

Selaginella apoda and *Selaginella moellendorffii* were grown for 60 days in gas perfusion chambers created from clear plastic Rubbermaid bins. Two to three of each plant were placed in the chambers for the duration of the experiment. One of these chambers had 10 ppm Ethylene perfused in, while the other had normal atmospheric air. The flow rate for both chambers was 10 ml/min. After 60 days were completed, tissue was harvested and used for sporangium counts and cell size measurements.

Sporangium Counts

After the perfusion period, strobili were harvested from *Selaginella apoda* and the ratio of megasporangia to microsporangia was recorded. With the aid of a dissection microscope, the sporangia were counted and recorded. Blank spaces on the strobilus and immature sporangia were not counted, while mature sporangia that had already ejected their spores were counted, because mature sporangia are able to be sexed, while immature ones are not.

Cell Size Measurements

Leaf tissue was also taken from *Selaginella apoda* and *Selaginella moellendorffii* so cell sizes could be measured. First, this tissue had to be fixed for viewing on the scanning electron microscope. First, the tissue was fixed in glutaraldehyde, followed by Sodium Phosphate washes. Then, the tissue was treated with osmium tetroxide, followed by an ethanol dehydration series. After this was finished, the tissue was critical point

dried and sputter coated, after which it was ready for viewing on the SEM.

Photographs were taken of the abaxial side of the major leaves. After scanning these images onto the lab computer, the cell sizes were measured with ImageJ64, with the results being tabulated in Microsoft Excel.

QRT-PCR ANALYSIS

RNA extractions were performed on entire 12d old *Arabidopsis* Columbia DR5::*GUS* seedlings with the Qiagen RNEasy Kit, following the manufacturer's instructions for plant tissue extraction. After quantification using a Nano-Drop, cDNA synthesis was performed using the Superscript III cDNA Synthesis kit from Invitrogen. qRT-PCR plates were set up using standard molecular biology techniques in a dead air hood, using Taqman probes/primer mix and complete master mix from Applied Biosystems, and then run on an Applied Biosystems StepOne Real Time PCR system (See table 3 for Taqman probe context sequences, from which the primers and probe were designed). Each plate setup had three biological replicates with one technical replicate apiece, and used *Arabidopsis* gene PDF1 ([AT3G25800](#)) as the endogenous control. Also, the original RNA from which cDNA was synthesized was added to wells and run with master mix to check for DNA contamination. After the runs were completed, each experimental well was normalized to the endogenous control, and mean Ct scores were found.

*EXAMINATION OF ACO GENE EXPRESSION PATTERNS USING PROMOTER::*GUS* FUSIONS*

Promoter Cloning

Bacterial artificial chromosomes (BACs) containing the 3' promoter region of

each ACO gene were ordered from Arabidopsis Biological Research Center and each BAC was isolated according to the BAC Prep/Clonal Analysis instructions accompanying the shipment of BACs. Each promoter region was subsequently amplified from its BAC using a two step PCR process: first, the reaction was run using Extaq, and then the same reaction was run, except PfuUltra enzyme was used, and the template for the second reaction came directly out of the first reaction. Table 2 summarizes the primers used. Once product was obtained from the PfuUltra reactions, the promoter regions were cloned into the pENTR/D-TOPO vector following the manufacturer's instructions. Next, the pENTR/D-TOPO vectors containing promoter regions were transformed via electroporation into *E. coli* cells, which were then plated on LB media with kanamycin resistance. Colony PCR was then performed on the resulting colonies, and colonies found to have a promoter insert were then grown in liquid kanamycin media so the plasmids could be extracted. Plasmid extractions were done using the QIAprep Spin Miniprep Kit, following the "Plasmid DNA purification using the QIAprep Spin Miniprep kit and a microcentrifuge." Next, an LR-Clonase reaction was run using the Gateway LR-Clonase kit from Invitrogen, using pENTR/D-TOPO and pMDC162, from the lab of Mark Curtis, University of Zurich, as the destination vector (Curtis and Grossniklaus, 2003). Next, an *Apa*I digest was carried out on the LR reaction to destroy the p-ENTR/D-TOPO but leave pMDC162 behind. The pMDC162 plasmids left from the LR reaction were then transformed into *E. coli* for colony PCR, and when suitable colonies were found, another plasmid prep was performed as before and the pMDC162 plasmids were then transformed into *Agrobacterium tumefaciens* strain AGL1. Freezer stocks were also made of promoter-carrying pMDC162 in *E. coli*.

Arabidopsis Transformation

Several weeks before transformation was to take place, *Arabidopsis* ecotype Landsberg erecta seeds were planted and groomed to a density of eight plants per punnett. About a week before transformation, the main inflorescences were cut near the base to stimulate axillary growth. When suitable axillary flowering had occurred, a 20 ml starter culture of pMDC162-containing *Agrobacterium tumefaciens* was begun and grown to maximum density. Then, one day before transformation, a large (approximately 500 ml) culture of the bacteria was grown from this starter culture to an OD 1.6-2. Once the proper OD is reached, the cells are collected by centrifugation (6.5k RPM at RT). They were then resuspended in an equal volume infiltration media (consisting of: 10mM MgCl₂, 5% sucrose, 1x B5 vitamins, 0.044μM benzylamino purine, and 0.03% Silwet L-77). When the bacteria were sufficiently resuspended in the infiltration media, they were poured into an appropriate vessel and each punnett was dipped in the bacteria for 5 minutes. The amount of media is enough for up to 8 punnetts, so long as care is taken not to soak up too much bacteria in the soil of the first few punnetts dipped. After the plants were done being dipped, they were laid on their side on top of paper towels in a tray and allowed to recover in an appropriate growth chamber.

Hygromycin Selection

pMDC162 carries hygromycin resistance, so once seeds were collected from the transformed *Arabidopsis* plants, hygromycin selection was carried out. First, the seeds were sterilized using ethanol and bleach, and then the seeds were plated on 0.6% phytagel plates containing 0.5X MS salts and 25 mg/L hygromycin. Next, the plates were wrapped

in foil and put in a drawer for 5-7 days to allow the plants to etiolate. When the foil was unwrapped, the plants that were etiolated were the hygromycin resistant plants. After 2-3 days greening on the lab bench, the resistant plants were transferred to non-hygromycin phytagel plates, and after about a week on those plates, finally transferred to soil.

GUS Staining

GUS staining was performed on brachts, rosettes, and flowers beginning with a fixation step in 90% acetone on ice for 20 minutes, and then the tissue was put in two washes of a 1:4 dilution of GUS stock buffer (1 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer pH 7.0, 50 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 50 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 10% Triton, 0.5 M EDTA) for 20 minutes each. X-Gluc staining was then performed. The X-Gluc stock solution was made of 25mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) in N-N Dimethylformamide, and then diluted to 40-60 μl of the stock solution into 1 ml of GUS working solution. This was done overnight at 37° C, after which the tissue was placed in 70% ETOH and pictures were taken using a dissecting microscope and a Nikon D-50.

STATISTICAL ANALYSIS

Means and SEs were determined by hand using Microsoft Excel or Open Office Calc. P-values were determined using a Student's T-test on GraphPad Quick Calcs.

Table 2: Primer Sequences

Target	Forward/Reverse	Sequence 5' → 3'
ACO1	Forward	CACCAGAAGAAGAAGTAGAAGAAGCAC
ACO1	Reverse	CTCACACACAGATTTGTTGAATGC
ACO2	Forward	CACCTACTTATCTTTGACTACGGAAC
ACO2	Reverse	CTCTCTCTCTTCTTTGAAAGTAATC
ACO3	Forward	CACCCTCTGACCCAAGGGTTTAATAGT
ACO3	Reverse	CTAGCTACTTAATGTTTTCGATCC
ACO4	Forward	CACCTATTTCCGCGGATTCTATCTTCG
ACO4	Reverse	GGGTTTCTTGGCCTAACTTTATG
ACO5	Forward	CACCGTGAGTTGTTCCAAGTAATGG
ACO5	Reverse	GTCAAAAAAGACTCTGTTTAGTAG

Table 3: List of context sequences for TaqMan Probes

PDF1	TTGATCAATCGGTTGTGGAGAAGAC
ACO2	ATGAATACAGGACGGCCATGAAAGA
ACO3	AATGAATACCGATTGGCAATGAAGG
ACO4	CGGCGATCAACTTGAGGTGATAACC

RESULTS

SELAGINELLA SPECIES RESPOND TO EXOGENOUS APPLICATION OF ETHYLENE

It has been observed that ethylene inhibits cell growth, so it was hypothesized that exposing *Selaginella* plants to exogenously applied ethylene would produce a similar

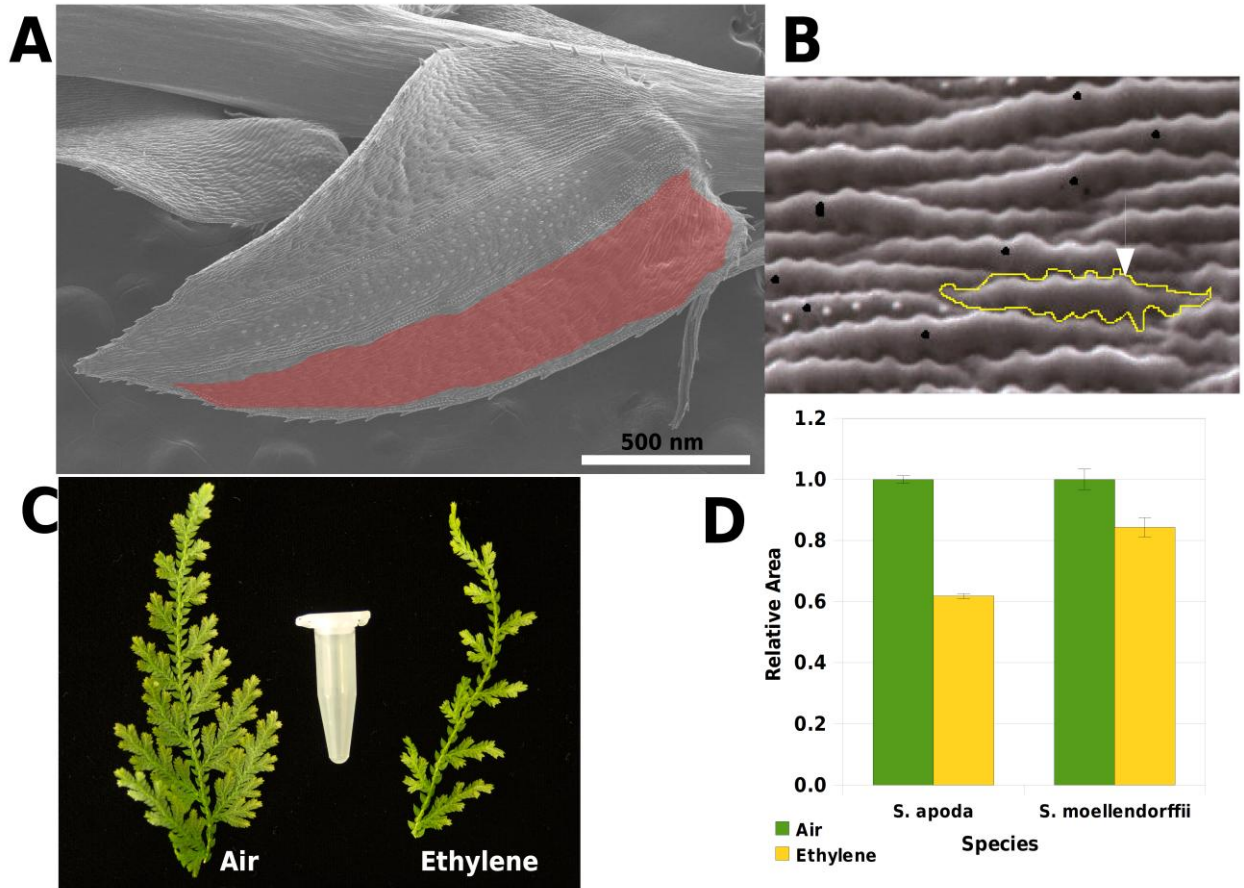
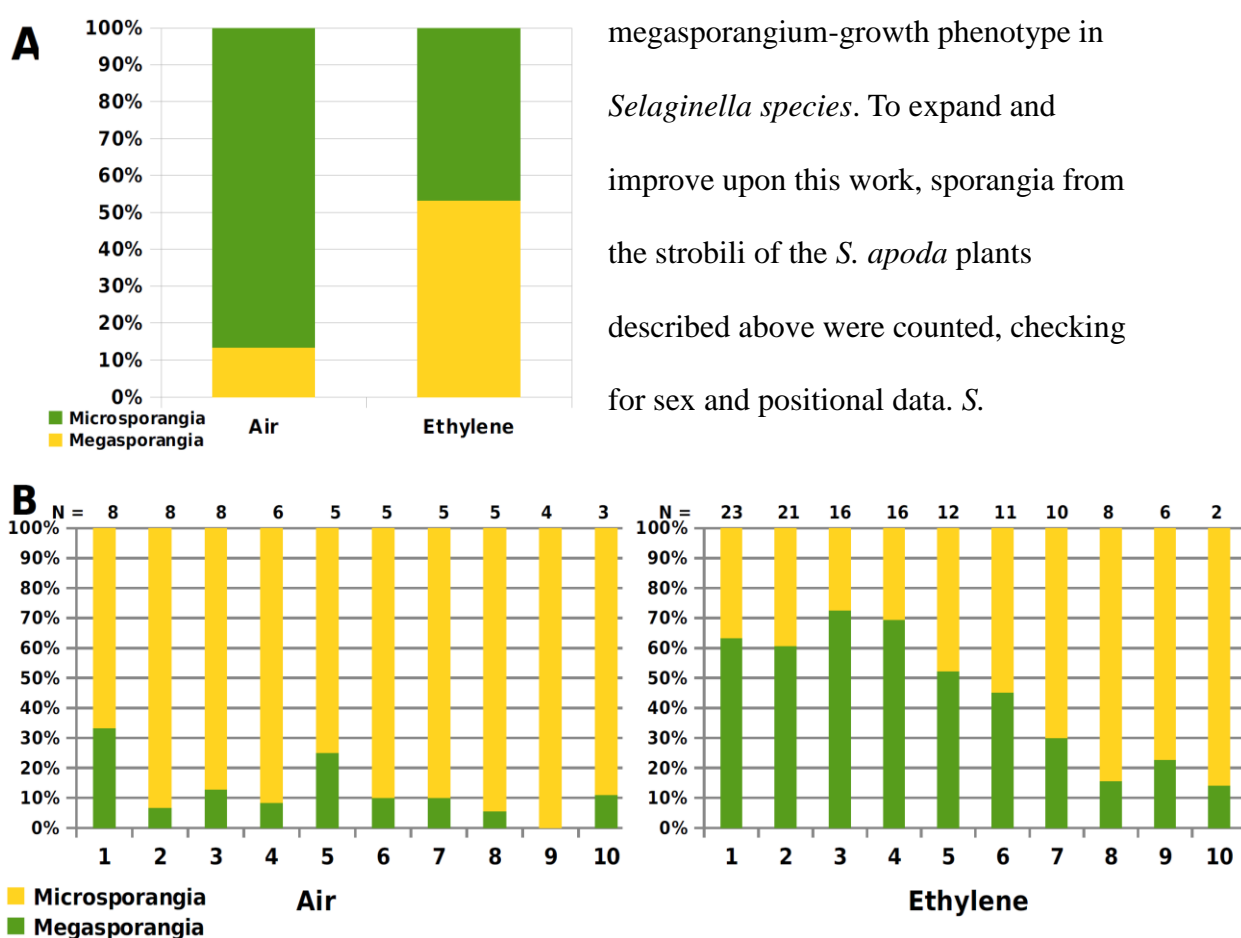


Figure 7: Selaginella Cell Size Measurements. A. *S. moellendorffii* major leaf, abaxial side. Red zones represent the lamella, the region where the measurements were made in both plants. B. Screenshot of measuring in process. In ImageJ, cells were traced freehand (arrow), the size was recorded, and the cell was marked. C. Air treated (left) and ethylene treated (right) branches show very different branching patterns, with the ethylene branches being much less dense. Centrifuge tube for scale. D. Relative areas of cells from *S. apoda* and *S. moellendorffii*. Error bars represent 2 SE and n=250 for each group.

They exhibit an alternating phyllotaxis on the branch. There are three main zones of cells on the abaxial side of the *Selaginella* major leaf. The middle region is marked by short, boxy cells and the presence of stomata. The margin of the leaf is made up of long, thin cells that have spike-like structures protruding from them. The region between the middle

and the margin is called the lamella, which is from where the cell size measurements were taken (Figure 7A and 7B). Branching patterns differed between the treatments. The ethylene-treated plants not only had fewer branches per unit area, there was also much less growth on the minor, axillary shoots (Figure 7C). As was expected, there was a decrease in cell size. In *S. apoda*, cell sizes from the ethylene-treated plants were 0.61 times the size of *S. apoda* cells grown in air. In *S. moellendorffii*, there is a similar decrease in cell size, with the ethylene cells being 0.84 times the size of the air cells (Figure 7D).

It has been shown that that exogenous ethylene application can induce an entirely



moellendorffii were not used because they do not produce megasporangia when grown in laboratory conditions. The results were consistent with previous work, in that the ratio of megasporangia to microsporangia increased significantly when plants were treated with ethylene, from 13.3% to 53.2% (Figure 8A). Positional data were also recorded, but there does not seem to be a difference in distribution of sporangia on the strobili (Figure 8B).

Many more strobili and sporangia were counted for ethylene-treated plants because there simply were more strobili and sporangia present. The differences in strobili numbers were not quantified, but in addition to megasporangia percentages having a sharp increase, an induction of strobilus growth may also be a response to ethylene application.

ANALYSIS OF ACO GENE EXPRESSION DOMAINS WITH GUS STAINING

To characterize the expression domains of ACO genes, we set out to design GUS transcriptional reporter fusions for each ACO promoter region into *Arabidopsis*. The promoter regions consist of the entire intergenic region 5' to each gene, including the 5' UTR. When the promoter regions were cloned into the final destination vector, pMDC162, transformation of *Arabidopsis* was then performed. The entire cloning and transformation process was completed with ACO1, which has yielded preliminary data. All of the other promoters except pACO4 have been amplified from their respective BACs and are awaiting further cloning work (Table 3). The work that has been completed has laid significant groundwork for substantial data to be gathered in the near future.

Gene Name	Amplify from BAC	P-ENTR/D-Topo	pMDC162	Transform <i>Arabidopsis</i>
ACO1	Y	Y	Y	Y
ACO2	Y	I	N	N
ACO3	Y	I	N	N
ACO4	N	N	N	N
ACO5	Y	Y	I	N

Y = Completed I = In Progress N = Not begun

Table 4: Progress on the construction and transformation of ACO Promoter-GUS fusions.

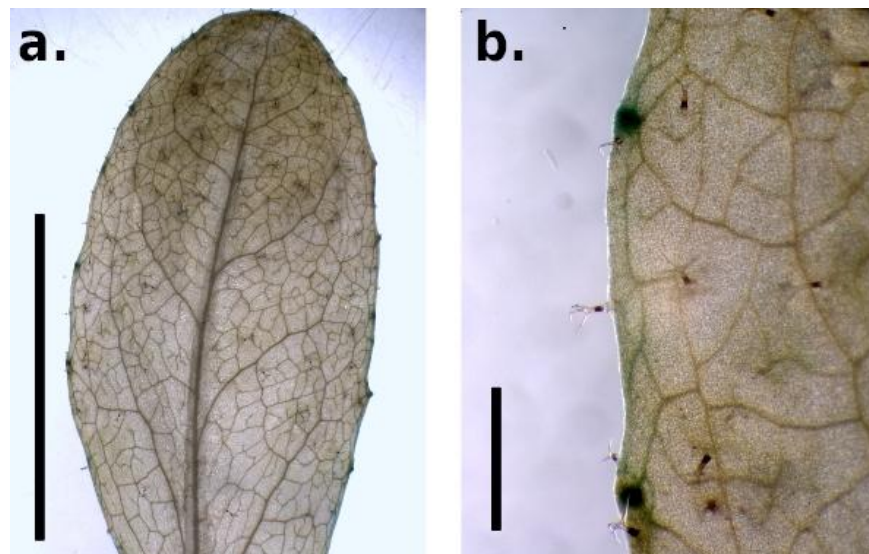


Figure 9: GUS staining of Arabidopsis rosette leaves. A. Whole leaf view. Note the punctate staining visible around the margins of the leaf. Scale bar is 1 cm. B. Close up of two of the stained points. Note the faint blue signal in the vasculature surrounding the points. Scale bar is 1 mm.

The completed pACO1::GUS fusion was transformed into *Arabidopsis* and hygromycin selection was performed to select transformants. The transformants were then allowed to grow in soil until mature. GUS staining was performed on rosette leaves, bracts, and flowers from the one mature line out of five total transformed plant lines obtained. On the leaves, especially rosettes, a distinct punctate staining pattern in the hydathodes, which are water secreting pores in the margin of *Arabidopsis* leaves, is visible (Figure 9). On some of the leaves, there was staining in the surrounding vasculature. There was also vasculature staining in the petals of one of the flowers.

ANALYSIS OF ACO GENE EXPRESSION LEVELS AND ACO NULL LINES BY qRT-PCR

The microarray database Genevestigator has helped to shape predictions for this study concerning gene expression of ACO genes. It is a repository of positional expression data in *Arabidopsis*, but also shows overall levels of expression at each major stage of development. To confirm and expand upon the data found in Genevestigator, qRT-PCR analysis was done to test wild-type gene expression levels. To perform the analysis, total RNA was extracted from all tissues of 12d old, light grown plants. When examining ACO3 expression levels as compared to ACO2 levels, it was expected that ACO3 expression would be much lower than ACO2 expression (Figure 10A). While ACO3 was expressed at lower levels than ACO2, the difference was not as drastic as Genevestigator indicated at this stage in development. As for ACO2 expression levels compared to ACO4 levels, qRT-PCR analysis revealed that ACO4 was expressed at a much higher level than ACO2 was (Figure 10B), which is the opposite of the prediction shaped by Genevestigator. These results highlight the importance of qRT-PCR

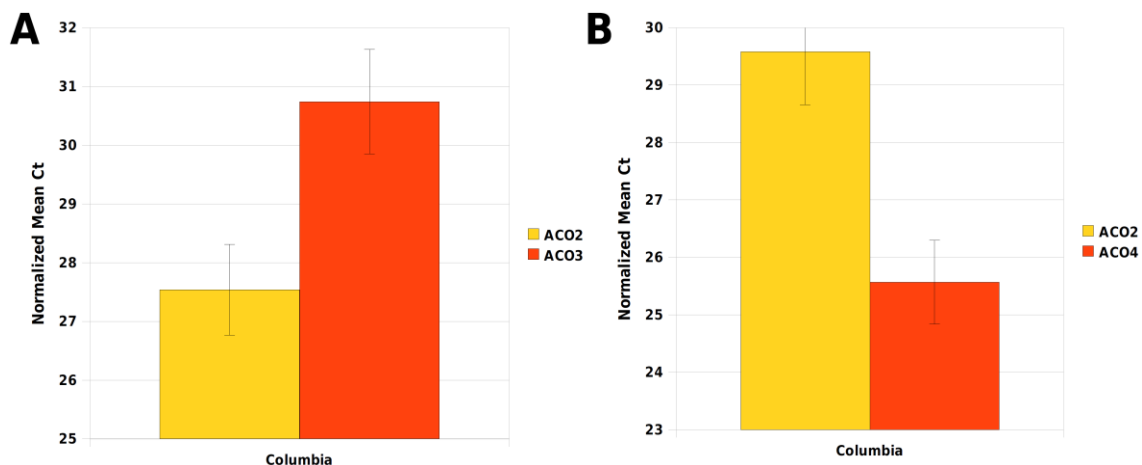


Figure 10: qRT-PCR analysis of wild-type levels of ACO gene expression. Error bars represent 2 SE. A. ACO2 levels compared with ACO3 levels. B. ACO2 compared with ACO4 levels.

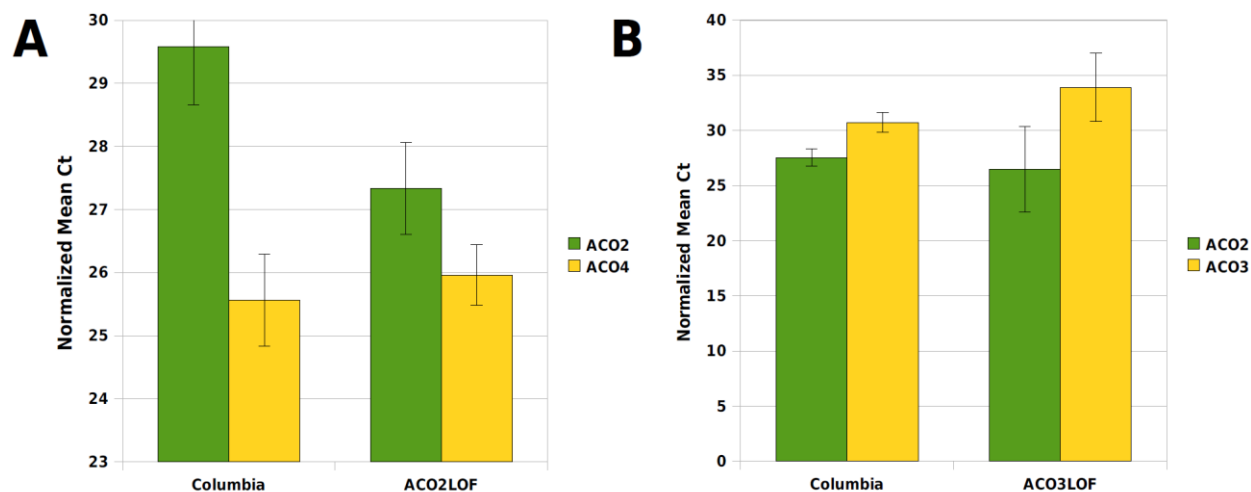


Figure 11: qRT-PCR analysis of ACO null lines. Results shown in terms of normalized mean Ct. Error bars represent 2 SE. ACO2LOF $p = 0.0343$. A. ACO2LOF background was tested, using probes for ACO2 and ACO4. B. ACO3LOF background was tested, using probes for ACO2 and ACO3. ACO3LOF $p = 0.0403$.

confirmation of microarray data.

Another aim of the qRT-PCR analyses was to examine the five ACO null plant lines obtained from the Ohio State Arabidopsis Biological Resource Center, to determine whether the null lines were actually nulls. When the ACO2LOF background was tested for ACO2 and ACO4 expression, it was found that ACO2 was upregulated in comparison to the wild type Columbia background. ACO4 levels stayed relatively constant (Figure 11A). In the ACO3LOF background, ACO3 levels were downregulated in comparison to the wild type background, yet still present. ACO2 levels were very slightly upregulated (Figure 11B).

DISCUSSION

SELAGINELLA ETHYLENE PERCEPTION AND RESPONSES

Ethylene signaling is a poorly understood process in basal plant lineages. While it is known that *Selaginella moellendorffii* possess homologs of ethylene signaling pathway genes, their actual functions have not been identified. What these experiments have shown is that *Selaginella apoda* and *Selaginella moellendorffii* do perceive ethylene, and that this perception precipitates a measurable response in the plants. The result that ethylene application decreases cell size in both *S. apoda* and *S. moellendorffii* is consistent with the expected result of a decrease in cell size. Perhaps this relates back to the function of ethylene in stress response pathways.

Is ethylene a hormone or a stress agent?

It is possible that these plants are perceiving such elevated environmental levels of ethylene as a stress response, instead of as a developmental cue. The reduction in cell size, the visually striking difference in color, and the reduction in leaf density between the two treatment groups supports this hypothesis. If the plant is perceiving ethylene application as a stress agent, it is possible that the ethylene is acting as a signal to reduce growth until conditions are more suitable, which would be signaled by a lack of ethylene for the plant to perceive. Also, the plants that were treated with ethylene take on a much more pale, almost yellow-green pigmentation that is vastly different from the deep green seen in the air treated plants. In effect, the plants appear stressed. While this color difference was not quantified here, it would be possible to do an ethanol extraction of the pigments in the leaves and analyze the extract on a spectrophotometer, thus quantifying

the difference in the pigment levels present in the plants during the experiment. Also, stunted minor branch growth in the ethylene treated plants could be an effect of the ethylene, as the plant could, once again, be perceiving the ethylene as a signal to reduce resource consumption and slow growth until conditions are better. Another assay that could be done to further explore the recovery of the plants would be to allow the plants to grow without ethylene for 30 days after the initial 60 day growth period with ethylene application, and measure their recovery in terms of pigment levels, leaf density observations, and cell size measurements. This still leaves the question of whether ethylene is a hormone or merely a stress agent open. To fully answer this question, ethylene production in *S. apoda* and *S. moellendorffii* needs to be demonstrated. If ethylene production cannot be demonstrated, it could be said to merely be a stress agent in these basal plants.

The sporangium counts from *S. apoda* tell a similar story. The number of strobili counted for the air plants is much smaller than the number of strobili counted for the ethylene plants. This was not quantified, because no unbiased assay could be devised for measuring strobili per unit area on the plant. However, quick observation of ethylene grown plants side-by-side with air plants makes it very clear that the ethylene plants are producing many more strobili, a phenomenon which could be called *hypersexuality*. This hypersexual response could be another stress response. If the plants are reducing resource use in other places, they could be doing so with the intention of funneling those extra resources toward reproduction, and thus making as many copies of the plant as possible before the plant dies. This could also explain the increased number of megasporangia. Normally, the limiting factor in *S. apoda* reproduction is the number of available

megaspores, because they are usually produced in very small numbers. The increase in megasporangium production could be explained as a move to flush the reproductive arena with more of the limiting factor so that reproduction is not as limited as it normally is- and perhaps so reproduction can take place in conditions that are not normally considered suitable.

Future directions:

Looking forward, the megasporangium ratios and strobili production could be useful as a visual, quantifiable phenotype in a genetic screen for ethylene-mutants in *S. apoda*. Because the difference in phenotypes is so distinct, ethylene-insensitive mutants could be spotted very easily in a mutant screen situation as plants grown in the presence of exogenous ethylene, but show no increase in megasporangium production or strobilus growth.

ANALYSIS OF ACO GENE EXPRESSION PATTERNS BY GUS STAINING

What are the expression domains of ACO genes in Arabidopsis?

Even though most of the molecular genetics research done with the ethylene biosynthesis pathway has been done in flowering plants, especially *Arabidopsis*, there is still much to be learned. ACO, the last enzyme in the canonical ethylene biosynthesis pathway, is relatively poorly understood in terms of its expression patterns in the plant and the functional redundancy of the five enzymes in the family. One of the main aims of this thesis was to elucidate the patterns of expression of the five ACO genes. Preliminary data show that there could be considerable distinctiveness in the function of the ACO genes. The data that were found show two things: first, that the transformation process

worked and that the lab now has truly transformed lines with the appropriate pACO1::GUS fusion; and second, that in rosette leaves, it appears that ACO1 is expressed solely in the hydathodes of the leaf. This expression is similar in pattern to expression shown by Aloni et al. (2003) with auxin promoter-GUS fusions. In that paper, auxin was shown to be expressed in the hydathodes of *Arabidopsis*, which form a punctate pattern around the margin of the leaf and secrete extra water.

The sole expression of ACO1 in the hydathodes also has broader implications. In ethylene biology, it is generally believed that the ethylene forming enzyme is constitutively expressed all over the plant. The evidence for restricted expression presented here contradicts that belief, setting up a possible paradigm change for how biologists think about ethylene in plants. This thesis research also has set up the completion of a comprehensive study of the spatial expression patterns of the ACO genes in *Arabidopsis*, similar to what has been shown with ACS genes. This information could illuminate a novel model of ethylene production. If the ACO enzymes show a high degree of specificity of function, i.e. they do not generally overlap in their expression domains on the plant, it would provide weight to the hypothesis that while ACS is globally the rate-limiting step of the ethylene biosynthesis pathway, ACO may be locally limiting. It is possible that ACC may be produced in remote parts of the plant and then transported through the xylem or the apoplast to its final destination cell, where it will then be converted to ethylene by ACO. This would solve the problem of ethylene transport. Since ethylene is a non-polar gas and does not go into aqueous solution easily, it cannot be transported through the plant in a controlled fashion. ACC on the other hand, goes into solution very easily, and can be readily transported through the plant in a controlled

manner. This would give the plant a much more precise point of control for the production of ethylene.

Future directions

This research will also help with the Engstrom lab's continued goal of performing a cross-species complementation assay on *Arabidopsis* in order to test the function of *Selaginella moellendorffii* ACO genes. If ACO gene expression can be well characterized in *Arabidopsis*, when the cross-species complementation assay is performed, the researcher will know what to expect in terms of levels of expression and ethylene production. The research done for this thesis has provided a solid base from which continued research will elucidate the patterns of ACO expression in *Arabidopsis*.

ANALYSIS OF ACO GENE EXPRESSION LEVELS AND ACO NULL LINES BY qRT-PCR

Microarray data, by their nature, are not as exact as qRT-PCR. They provide a reasonable starting point for hypothesis formation, but are not exact enough to be the only evidence supporting gene expression patterns. GUS staining is also not very useful for quantitative studies of gene expression. It can yield excellent spatial resolution, but it is very difficult to quantify. Because of these reasons, qRT-PCR studies were undertaken to determine the relative levels of gene expression in *Arabidopsis* wild type Columbia seedlings and to determine whether *Arabidopsis* ACO null lines were actually knock outs of the gene.

Examination of endogenous ACO gene expression:

Experiments such as those reported in this thesis show the value in validating microarray results with qRT-PCR data. The microarray data from Genevestigator suggested that ACO2 would be very highly regulated at the seedling stage, which is comparable to 12d old plants. It also showed that ACO4 expression would still be high, but not as high as ACO2. The qRT-PCR results are just the opposite. They show ACO4 being more highly expressed than ACO2. A similar result was found when ACO2 and ACO3 levels were tested at the same time. ACO2 is predicted to be much more highly expressed than ACO3 at the seedling stage. It was seen that ACO3 is expressed at lower levels, but not at the extremely low levels predicted by Genevestigator. These results are very important, because they not only give a better idea of what relative gene expression levels actually look like in these plants at 12d old, but they are improving upon the substantial amounts of microarray data by giving more accurate gene expression data.

Examination of ACO null lines for knockout gene expression

The data from the examination of the ACO2LOF and ACO3LOF null lines are much different than expected. The hypothesis was that there would be no expression of the gene that had been knocked out, but instead, there was still expression of both ACO2 and ACO3 in their respective knockout lines. In the ACO2 line, the ACO2 signal shows upregulation over the wild type, by a significant amount. After looking at sequence data for the ACO2 gene and the location of the insert, it was determined that the TaqMan probe for ACO2 lies 3' of the insert. This is a strange problem to be having, because the T-DNA insert should be stopping transcription before it can transcribe the entire gene. The most likely possibility is that there is a transcriptional start in the T-DNA, which is

actively transcribing the ACO2 gene and causing a signal to show up where there should be none. If that is not the case and the gene is being transcribed from the original start of transcription, some questions are raised. For upregulation to be taking place, the nonfunctional ACO2 protein would have to somehow be feeding back on itself, a process that is not known as of yet.

ACO3 poses the same problems. The qRT-PCR experiments show definite presence of ACO3 transcripts, even though the ACO3LOF background should have none. As with the ACO2 TaqMan probe, the ACO3 probe lies 3' to the T-DNA insert, meaning it binds downstream of where transcription should be stopping, and thus no signal should be seen. Once again, it is possible that there is a transcription start in the T-DNA insert and that transcription is only taking place on the 3' end of the gene. Another problem for the ACO3LOF tests is that when the RNA that was the source of the cDNA in the ACO3LOF wells was tested, there was amplification in some of the RNA wells, where there should have been none. This means some sort of DNA or cDNA contamination had gotten into the well and been picked up by the probes. This contamination could be part of why ACO3 expression was detected in the LOF background, but it is not a sufficient explanation, as the amplification in the RNA wells was only slightly above the levels that could be considered negligible.

Because of time constraints and small sample sizes, adequate data were not able to be collected for these experiments, making proper statistical analysis impossible. Thus, the data are expressed in normalized mean Ct scores, which allow for the data to be explained and understood in the short term. In the long term, more data need to be accumulated and correct statistical analysis performed.

CONCLUSIONS

This thesis sheds light on the evolutionary path of ethylene biology in lower plants and the way we understand the synthesis of ethylene in higher plants. This research is very important from a number of standpoints. First, it helps to draw a clearer path of the evolutionary route that ethylene in plant biology has taken. Also, learning the expression patterns of ACO genes will make possible a greater understanding of this very important process in plant development and physiology. Like any good science, this research raises as many questions as it answers and has provided a solid base from which to continue further forays into the field of ethylene biology.

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