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Ethylene Insensitivity in Maize:
Analysis of Ethylene Receptors and the Ethylene Response in Maize

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
University of California, Riverside, March 2011
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The biological role of ethylene, $H_2C=CH_2$, was first identified as a plant hormone responsible for leaf drop from the observation that plants relatively close to gas lamps lost their leaves. Later, it was then known as an important gaseous hormone for climacteric fruit ripening. Further research revealed that ethylene not only regulates entry into several types of plant developmental cell death and senescence programs, but also mediates plant responses to biotic and abiotic stress. For example, ethylene has been implicated in promoting kernel abortion under shading stress in maize. Ethylene production is controlled by the nutritional and stress status of a plant. Despite the broad range of ethylene's effects on development, the primary steps in ethylene action are assumed to be similar in all cases: They all involve the binding of ethylene to a receptor, followed by activation of one or more signal transduction pathways leading to the cellular response. Ultimately, ethylene exerts its effect primarily through alterations in the pattern of gene expression.

Although the hormonal control of root growth and development has been extensively studied, relatively little is known about the role that ethylene plays in cereal root development. To understand how the ethylene biosynthetic machinery is spatially regulated in maize roots and how changes in its expression alter root growth, the expression of ACC synthase (encoded by *ZmACS2*, *ZmACS6*, and *ZmACS7* in maize) was observed in the root cap and in cortical cells whereas the expression of ACC oxidase (encoded by *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35* in maize) was detected in the root cap, protophloem sieve elements, and the companion cells associated with metaphloem sieve elements. The results suggest that expression of *ZmACS6* is important in regulating growth of maize roots in response to physical resistance.

To date, many studies on ethylene insensitivity have focused on the function of the Arabidopsis dominant-negative mutant ethylene receptor gene (*etr1-1*) in Arabidopsis or other species. To understand more about the effect of ethylene on cereal crops, maize dominant-negative ethylene receptors (e.g. *Zmetr2* and *Zmers1*) were generated by altering a conserved cysteine residue in one of the transmembrane domains in the N-terminal region of the receptor. Taking the advantage of its short generation time, *Zmetr2* and *Zmers1* were first studied in Arabidopsis. The results suggest that Cys65 in maize ZmERS1 and ZmETR2 plays the same role that it does for Arabidopsis receptors. Moreover, the results demonstrate that the mutant maize ethylene receptors are functionally dependent on subfamily 1 ethylene receptors in Arabidopsis to exert their

dominance, indicating substantial functional conservation between maize and Arabidopsis ethylene receptors despite their sequence divergence.

The *etr1-1* mutant confers a state of ethylene insensitivity constitutively during the lifetime of a plant. Therefore, the effect of *etr1-1* on plant growth and development is limited in that it does not reveal what roles ethylene might play in specific cell types or developmental stages separate from its global influence on the plant. To study the role of ethylene in specific organs or at certain developmental stages in maize, expression of the maize dominant-negative ethylene receptor, *Zmetr2*, was driven by organ-specific promoters, i.e., from PEPC (Phosphoenolpyruvate carboxylase) and RbcS (Rubisco small subunit), for leaf-specific expression, and SH1 (Shunken1), for kernel-specific expression in transgenic maize. Such an approach allows the examination of the effects of creating a state of ethylene insensitivity in specific organs to determine the role of ethylene under normal growth conditions or conditions of stress.

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CHAPTER 1

INTRODUCTION

The biological role of ethylene was first discovered in 1901 by Neljubov, who found that ethylene was the active component of illuminating gas that caused the horizontal growth of etiolated pea seedlings. Since then, numerous physiological effects of ethylene on a variety of plant species have been discovered (reviewed in 1). During germination, ethylene causes the hypocotyl to swell and broaden, girding it as it penetrates through soil. As the plant matures, ethylene can influence sex determination and promote fruit ripening, depending on the species. Ethylene has effects not only on the development of the plant, but also on the communication between the plant and its surroundings. Wounding, pathogenic attack, flooding, fruit ripening, development, senescence, and ethylene treatment itself can induce ethylene production. The plant, by responding to the induced ethylene, will be able to proceed to the next stage of development or respond to a challenging environment. For instance, stress conditions, e.g., shading, drought, and heat, are perceived by leaves of maize and then communicated to the ears to induce kernel abortion at the ear tip, resulting in fewer kernels per ear (2-7).

That ethylene is a plant hormone was established when it was found to be produced by plants and to affect plant growth and development at very low concentrations. The pathway for the biosynthesis of ethylene, the Yang cycle, was identified by Yang in 1984 (reviewed in 8, 9). The precursor of ethylene

biosynthesis is the amino acid, L-methionine. After the addition of an adenosine, L-methionine is converted to S-adenosyl methionine (AdoMet). The conversion of S-adenosyl methionine to ACC (1-aminocyclopropane-1-carboxylic acid) is the first committed step of ethylene biosynthesis, and this reaction is catalyzed by the enzyme ACC synthase. ACC is efficiently converted to ethylene by ACC oxidase, and then the ethylene produced can bind to receptors, which are similar to two-component regulators (10). The binding of ethylene to the receptor can change the interaction between the receptor and CTR1, a Raf-like kinase (11, 12). The inactivated CTR1 will inhibit the repression of ethylene response and in turn alter gene expression.

There are five different ethylene receptors in Arabidopsis, ETR1, ERS1, EIN4, ETR2, and ERS2 (13, 14). The ethylene receptor family shows characteristic features of an N-terminal ethylene-binding transmembrane domain, a GAF protein-protein interaction domain, a histidine protein kinase domain, and, for in some receptors, a receiver domain. These five receptors fall into two subfamilies based on His protein kinase domain: ETR1/ERS1, and ETR2/EIN4/ERS2. Subfamily I members, ETR1 and ERS1, have all five typical His protein kinase motifs (H, N, G1, F, and G2), while subfamily II members, ETR2, EIN4 and ERS2, lack most of the consensus motifs of a His protein kinase. These five ethylene receptors could also be grouped into two classes by their C-terminal domains. ETR1, ETR2, and EIN4 have an additional C-terminal domain, while ERS1 and ERS2 do not. The C-terminal domain is also called a receiver

domain, because in bacteria, this domain is capable of relaying the phosphate group from the histidine kinase domain to the response regulator, usually via another protein. Ethylene receptors are similar to two-component regulators in bacteria (10). In response to a given stimulus, the two-component regulators will autophosphorylate its conserved histidine residue. The phosphoryl group is transferred to a conserved Asp residue in the receiver domain, and is then transferred to a response regulator. In the two-component system, the histidine kinase domain senses a signal input and the response regulator mediates the output. Although ethylene receptors are related to bacterial two-component regulators, the phosphorylation in histidine kinase domain is not required for ethylene signal transmission (15-20).

In maize, only two kinds of ethylene receptors are found, ZmETR2 and ZmERS1 (21). Each kind of receptor is encoded by two genes: *ZmERS1-14* and *ZmERS1-25* for ERS1 ethylene receptors, and *ZmETR2-40* and *ZmETR2-9* for ETR2 ethylene receptors. Like Arabidopsis ETR1 and ERS1, ZmERS1 contains three N-terminal hydrophobic regions that serve as transmembrane domains; Cys-4 and Cys-6, which are required to form homodimers; Cys-65 and His-69, implicated as residues for Cu(I)-binding needed for binding of ethylene; and the histidine protein kinase domain with all five H, N, G1, F and G2 motifs. However, ZmERS1 lacks the receiver domain which is present in Arabidopsis ETR1 but absent in ERS1. Thus, ZmERS1 is ERS1-like rather than ETR1-like. ZmETR2 contains a fourth transmembrane region and lacks the N, G1, F and G2

consensus motifs characteristic of ETR2/EIN4/ERS2 receptors. ZmETR2 has a receiver domain like ETR2 and EIN4. Moreover, ZmETR2 contains a serine stretch instead of the F consensus motif and lacks the histidine site, making this receptor to be like Arabidopsis ETR2, but unlike EIN4.

Ethylene receptors are localized in the ER membrane (22). Ethylene binds to membrane spanning regions in the sensor domain of receptors (23, 24). In the absence of ethylene, the receptors are predicted to remain in a functionally active state, which is able to interact with CTR1 (12). CTR1 is activated by association with the ER-bound receptors and represses downstream ethylene responses by a mechanism that requires its C-terminal Ser/Thr kinase domain. In the presence of ethylene, ligand binding actually changes the interaction between the receptor and CTR1, and relieves the repression of downstream ethylene responses (11, 12). As a result, EIN2 is activated and a transcriptional cascade involving the EIN3/EIL and ERF transcription factors is initiated (17). Both families of transcription factors are involved in regulating ethylene responses. CTR1 is a negative regulator of the ethylene responses, as its loss-of-function mutants have constitutive ethylene responses (11). It is a Raf-like Ser/Thr kinase with similarity to a mitogen-activated protein kinase kinase kinase (MAPKKK), suggesting the involvement of a MAP-kinase-like signaling cascade in the regulation of ethylene signaling. Although there are multiple MEKs and MAPKs existing in plants, none have been directly implicated in ethylene signaling to date (25-28).

As complicated as a hormone can be, environmental factors determine the plant's response to ethylene (29), and different parts of the plant respond differently to the same level of ethylene (2, 5, 30, 31). The signal of ethylene can be modulated by the distribution of isoform genes in different organs or tissues (13, 32-34). For example, ACC synthase and ACC oxidase have several isoforms, and the expression of those isoforms is regulated spatially and temporally (33, 34). Some isoforms are positively regulated by ethylene, and some are negatively regulated. In addition, there are different gene expression levels in different organs or tissues. It was found that higher expression of receptor genes occurs in reproductive organs and tissues (32-36). Higher expression of receptor genes is believed to make the cell less sensitive to ethylene, because in the presence of a high level of receptors, higher levels of ethylene are required to release the suppression. Ethylene is a hormone, and as a hormone, it has a complicated network with many other signaling pathways. Therefore, specific responses of different parts of the plant can be carried out precisely by ethylene signaling (21, 32-37).

A dominant mutant in Arabidopsis, *etr1-1*, was first identified by Bleecker et al. in 1988 and found to be insensitive to ethylene in all the ethylene responses analyzed (23). Etiolated *etr1-1* seedlings are elongated with thin hypocotyls in the presence of ethylene rather than short and thick as observed in wild-type seedlings. The *etr1-1* mutant exhibits delayed flower senescence and abscission, lower seed weight, slower seed germination, less efficient rooting and delayed

growth of seedling, and enhanced ethylene production in pollinated flowers (23, 29). These changes in *etr1-1* plants have helped to elucidate the role of ethylene in plants.

ETR1 acts upstream of the ethylene signal transduction pathway. The ETR1 receptor contains a novel amino-terminal domain that possesses ethylene-binding activity (23, 24). The *etr1-1* mutant contains a Cys65Tyr mutation in the second hydrophobic domain of the transmembrane region (10). This mutation disturbs the binding of the copper cofactor (Cu), which is needed for ethylene binding (38). Therefore, even when ethylene is present, *etr1-1* plants are unable to perceive and respond to ethylene. ETR is one of five ethylene receptors in Arabidopsis, and the *etr1-1* mutation is sufficient to repress the signaling cascade. *etr1-1* is a dominant mutation in Arabidopsis, and the expression of *etr1-1* in other species can confer ethylene insensitivity. For example, constitutive expression of Arabidopsis *etr1-1* gene delays fruit ripening in tomato and senescence of flowers in petunia (39). With the *etr1-1* mutant, the effect of ethylene and its signal transduction pathway in Arabidopsishas been extensively studied. However, considerably less in known about receptor functions in maize.

The following work represents an examination of the function of ethylene in maize. First, the regulation of tissue-specific expression of the ethylene biosynthetic machinery in root growth was studied. ACC synthase (*ZmACS*) expression was observed in the root cap and in cortical cells whereas ACC

oxidase (*ZmACO*) expression was detected in the root cap, protophloem sieve elements, and the companion cells associated with metaphloem sieve elements. Second, the function of the two maize ethylene receptors, ZmETR2 and ZmERS1, was analyzed in Arabidopsis. A Cys to Tyr mutation was introduced at amino acid 65 in the transmembrane domain of ZmERS1 and ZmETR2 to generate mutant maize receptors that copy the mutation present in the etr1-1 dominant negative mutant. As a result, the Cys65 residue in maize ZmERS1 and ZmETR2 was observed to play the same role that it does in Arabidopsis receptors. A similar degree of ethylene insensitivity was conferred by full-length *Zmetr2* and *Zmers1* or by expression of the N-terminal portion of *Zmers1*. Moreover, the mutant maize ethylene receptors were functionally dependent on subfamily 1 ethylene receptors in Arabidopsis. Finally, the effect of expression of the dominant negative ethylene receptor gene, *Zmetr2*, on ethylene responses in maize was examined. To this end, *Zmetr2* expression was directed by organ-specific promoters, i.e. Rubisco (*RbcS-m3*), phosphoenolpyruvate carboxylase (*PEPC*) and Shrunken1, so that, the ethylene insensitivity was restricted to the leaf or kernels during certain developmental stages. The expression patterns of *Zmetr2* were characterized in different organs, e.g. leaf, husk leaf, silk, etc., at different developmental stages, and insensitivity to ethylene confirmed by the lack of induction of ethylene-inducible genes.

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CHAPTER 2

Tissue-Specific Expression of the Ethylene Biosynthetic Machinery Regulates Root Growth in Maize

ABSTRACT

Although the hormonal control of root growth and development has been extensively studied, relatively little is known about the role that ethylene plays in cereal root development. In this work, how the ethylene biosynthetic machinery is spatially regulated in maize roots and how changes in its expression alter root growth have been investigated. ACC synthase (*ZmACS*) expression was observed in the root cap and in cortical cells whereas ACC oxidase (*ZmACO*) expression was detected in the root cap, protophloem sieve elements, and the companion cells associated with metaphloem sieve elements. Roots from *Zmacs6* mutants exhibited significantly reduced ethylene production, a smaller root cap of increased cell number but smaller cell size, accelerated elongation of metaxylem, cortical, and epidermal cells, and increased vacuolation of cells in the calyptrogen of the root cap, phenotypes that were complemented by exogenous ACC. *Zmacs6* mutant roots exhibited increased growth when largely unimpeded, a phenotype complemented by exogenous ACC, whereas loss of *ZmACS2* expression had less of an effect. In contrast, *Zmacs6* plants exhibited reduced root growth in soil. These results suggest that expression of *ZmACS6* is important in regulating growth of maize roots in response to physical resistance.

INTRODUCTION

As a phytohormone, ethylene has been shown to be involved in germination, seedling triple response, sex determination in some species, stem elongation in deepwater rice, senescence of leaves and flowers, fruit ripening, cereal endosperm programmed cell death, organ abscission, and pathogen responses (1-6). Ethylene also regulates root growth, adventitious root formation, root hair growth and development, gravitropism, and root remodeling in response to adverse environmental conditions such as hypoxia or mechanical impedance (7-14).

As it does in many plant species, moderate to high levels of exogenous ethylene inhibit root elongation in maize (15, 16). The inhibitory effect of ethylene on root growth is rapid, occurring within 20 min and largely inhibited cortical cell elongation in the region just distal to the root apex similar to observations made in other species (15). Maize roots treated with inhibitors of ethylene perception exhibited slightly enhanced growth, suggesting that the endogenous level of ethylene produced during normal root growth may serve to reduce root growth (15). Ethylene insensitive tomato also exhibited increased root mass under normal growth conditions (11). Low levels of ethylene (below 0.1ppm) promote root growth (7), suggesting that the level of ethylene determines whether it serves a stimulatory or inhibitory role.

The ethylene-mediated reduction in root growth, which is often accompanied by radial expansion, increases the ability of roots to overcome the physical resistance presented by some soils. Roots from tomato seedlings treated with inhibitors of ethylene perception failed to penetrate 2% agar but grew normally on 0.5% agar (9). Ethylene-insensitive tomato roots also exhibited poor penetration in response to mechanical impedance (11). Although ethylene often has an inhibitory function, it can serve a positive role in plant growth and development, for example in promoting adventitious root formation, root hair development, and hypocotyls elongation (11-13, 17). Ethylene insensitive tomato exhibited reduced adventitious root formation and reduced root hair length relative to wild-type roots (11). Despite the role that ethylene plays in regulating root development and growth, little work has been done to identify those specific gene family members involved in ethylene biosynthesis that are needed to regulate root growth.

Ethylene is produced from methionine that is first converted to S-adenosylmethionine (AdoMet) by S-adenosylmethionine synthase. AdoMet is then converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). ACC oxidase (ACO) generates ethylene by oxidizing ACC in a reaction that also produces CO_2 and HCN (18). ACS and ACO are encoded by multigene families in plants. Twelve *ACS* genes from Arabidopsis have been isolated and characterized (19, 20). Cell specific and overlapping expression of *ACS* promoter::reporter fusion constructs was observed in Arabidopsis, including roots

(21). All members, except two, are expressed in the vascular tissue of the maturation zone of the root and only one, i.e., *ACS8*, is also expressed in the root cap (21). Two of the five members of the *ACS* gene family in rice have been implicated in the growth response following submergence (22, 23) in which the expression of one, *OS-ACS5*, has been examined in detailed. Expression of *OS-ACS5* was detected mainly in vascular tissues in stems and young leaves of air-grown plants and was induced by submergence (24). Expression of *OS-ACS5* was also detected in the elongation zone of lateral roots (24). Expression profiling of members of the ACC oxidase gene family has received considerably less attention (25, 26) despite the fact that it is the ethylene forming enzyme. Therefore, it is not known whether the *ACS* expression in vascular tissue of Arabidopsis or rice produces ACC for transport to other regions or whether these tissues themselves are fully competent to produce ethylene.

The *ACS* gene family in maize (*ZmACS*) contains at least three members, i.e., *ZmACS2*, *ZmACS6*, and *ZmACS7* whereas the *ACO* gene family (*ZmACO*) contains four members, i.e., *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35* (27). Because of the smaller size of the *ACS* gene family, knockout mutants were isolated to examine the roles of ethylene during maize growth and development (28). Loss of one member, *ZmACS6*, resulted in a reduction of up to 90% of foliar ethylene production and a substantial delay in leaf senescence which could be reversed by the exogenous application of ACC. *Zmacs6* plants were characterized by delayed leaf senescence, increased photosynthetic function,

and greater drought tolerance. *Zmacs2* plants exhibited a 45% reduction in foliar ethylene production and an intermediate delay in leaf senescence. All leaves examined, including those young and fully-expanded leaves that were far from senescing, exhibited improved photosynthetic function, suggesting that in addition to a role in regulating leaf senescence, ethylene may control aspects of leaf development that are independent of senescence. The role that ethylene may play in other aspects of maize growth and development, such as in roots, has received less attention.

In this report, the role of ethylene during root growth and development has been investigated. Of the three *ZmACS* gene family members, *ZmACS6* was expressed in the root cap as well as in the root proper as measured by quantitative RT-PCR (qRT-PCR). *ZmACS2* and *ZmACS7* were poorly expressed in the root cap but their expression increased in the elongation zone of the root. In situ RNA localization analysis revealed expression of *ZmACS6* in the root cap and outer cortex whereas expression of the *ZmACS2/ZmACS7* subfamily was largely restricted to the inner cortex. The four members of the *ZmACO* gene family were expressed in the root cap and also in the elongation zone of the root. Expression of one *ZmACO* subfamily (i.e., *ZmACO15/31*) was restricted to the root cap and protophloem sieve element (PSE) whereas expression of the other subfamily (i.e., *ZmACO20/35*) was confined to the root cap and the companion cells (CC) associated with the protophloem. Roots from *Zmacs2-1/Zmacs6-1* double mutant plants, in which ethylene production was reduced by 85%,

exhibited a smaller root cap containing more cells of smaller size, earlier elongation of metaxylem, cortical, and epidermal cells, and increased vacuolation of cells in the calyptrogen of the root cap. The loss of *ACS* expression also resulted in an increase in the size of mature root cells. *Zmacs6-1* roots exhibited a higher rate of growth when largely unimpeded but reduced growth in the presence of mechanical impedance. Reduced root biomass accumulation was also observed in soil-grown *Zmacs6-1* plants, indicating that *ZmACS6* was responsible for maintaining root growth in soil. Results suggest that *ZmACS6* expression is important in regulating growth of maize roots in response to physical resistance.

MATERIALS AND METHODS

Plant growth conditions

Kernels of wild-type B73 and *Zmacs* mutants were sown in vermiculite and watered on the first and third day. Duplicate pots were watered with 10 µM ACC beginning on the third day. The apical 1 cm of roots was collected after 5 days of growth and fixed overnight with 2 % glutaldehyde and paraformaldehyde in 75 mM phosphate buffer, pH 7.2. The roots were dehydrated in an ethanol series, infiltrated and embedded in JB-4 (Polysciences, Inc.). Three µm longitudinal sections were collected using a Hacker 5030 microtome and stained with 1% Aniline Blue Black (for protein staining) and in some cases counterstained with

Periodic Acid Schiff (for carbohydrate staining). Digital images of median sections were collected using a Leica microscope with brightfield optics. The size of the root cap, root cap cell number, and the number of peripheral cells in the root cap were determined from median longitudinal sections. The total areas of the quiescent center (QC) and calyptrogen were traced separately and measured. The area made up by cell walls, extracellular space, plastids, cytoplasm and nucleus stained by Aniline blue black was measured as was the area occupied by vacuoles that remained unstained. The percent space occupied by vacuoles was determined from the fraction of vacuolar to total cell area. The first five cells of the developing vessel elements next to the QC were traced individually and their longitudinal axis (i.e., length) and transverse axis (i.e., width) were measured using MCID Elite software v. 7.0. Cortical and epidermal cells in the root proper just behind the root cap were similarly measured.

Quantitative RT-PCR

Total nucleic acid was isolated from roots from one week old seedlings using the protocol as described (28). Following the initial precipitation and resuspension in TE, total RNA was further purified by 2 rounds of $LiCl_2$ precipitation according to methods described by Sambrook et al. (29). 50 µg total RNA was treated with RQ1 DNase (Promega) to ensure that no contaminating DNA was present. Two µg of total RNA was used directly for cDNA synthesis

using the Omniscript RT kit (Qiagen) with oligo-dT20 as the primer. Analysis of transcript abundance was accomplished using the QuantiTect SYBR Green PCR kit (Qiagen). Reactions contained 1X buffer, 0.5 µl of the reverse transcription reaction (equivalent to 50 ng total RNA) and 0.25 µM (final concentration) forward and reverse primers in a total reaction volume of 25 µl. Reactions were carried out using an ABI PRISM 7700 sequence detection system under the following conditions: 95ºC/15 min (1 cycle); 95ºC/30 sec, 62ºC/30 sec, 72ºC/2 min (50 cycles); 72ºC/5 min (1 cycle). Each gene was analyzed a minimum of four times and the average and standard deviation reported. All primer combinations were initially run and visualized on an agarose gel to confirm the presence of a single product of the correct size. All amplification products were subcloned into the pGEM-T Easy vector system (Promega) and used for the generation of standard curves to facilitate conversion of the expression data to the number of copies/µg RNA basis. For semi-quantitative RT-PCR, 95ºC/15 min (1 cycle); 95ºC/30 sec, 58ºC/30 sec, 72ºC/2 min (28-31 cycles); 72ºC/5 min (1 cycle) was used.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ZmACS2	ATCGCGTACAGCCTCTCCAAGGA	GATAGTCTTTTGTCAACCATCCCATAGA
ZmACS6	AGCTGTGGAAGAAGGTGGTCTTCGAGGT	AGTACGTGACCGTGGTTTCTATGA
ZmACS7	ATCGCGTACAGCCTCTCCAAGGA	CAACGTCTCTGTCACTCTGTGTAATGT

ZmACO15	CTCGTCTTCGATCAATTCCCAAGT	TACATTATCATTATTTCTCCGGCTGT
ZmACO20	CTCATCCTGCTGCTCCAGGACGAC	TCCACGATACACGCATAACCACCGT
ZmACO31	CTCGTCTTCGATCAATTCCCAAGT	ATAGCAAAGAGGGCAACTAGCTAGT
ZmACO35	CTCATCCTGCTGCTCCAGGACGAC	ACACACATAACTGTGCCACTATAAGCA
ZmERS1	GGCTGCAGTCCGTGTACCTCTTC	GTTCAAGGCTTCCATCCTCGAG
ZmETR2	AGGAGTCTCAGTCGATCGGAGAG	CCCAGCTGCACAATCTTCTTGCACATATTGAAGC
ZmERF1	GACATCGACGCATCCCATATCTATAGG	GCGCTCCACGGGAAAGTTGAGCACG
ZmXET	CGTTGATTCTGGCGGCGGTGCTGC	GTCGAACCACATCCGGAACTGCTGC
eIF4A	CATGCCCCCTGAGGCCCTTGAG	AGCAGGTCGGTGGTGATGAGCAC

RNA in situ localization

RNA in situ localization was carried out as described previously (30) with modifications. Roots were placed into FAA (50% EtOH, 10% formalin, 5% acetic acid), vacuum infiltrated, and stored for two days at 4ºC. The fixative was replaced with 70% EtOH and the samples dehydrated through an EtOH series (85%, 95% and 100%) at 1-day intervals at 4ºC. Ethanol was replaced with Hemo-De through a graded series [2 hr 50% EtOH: 50% Hemo-De (Fisher), three treatments in 100% Hemo-De for 2 hr]. Samples were then infiltrated in increasing concentrations of Paraplast Plus, embedded in 100% Paraplast Plus, sectioned on a rotary microtome (15 µm thick), and fixed on Probe-On-Plus

slides (Fisher). Sections were treated as described by Jackson (31) with modifications. Sections were deparaffinized in 100% Hemo-De, rehydrated through an EtOH series, equilibrated in PBS, deproteinized with proteinase K, treated with glycine and washed twice in PBS. Sections were post-fixed with 4% paraformaldehyde, acetylated with acetic anhydride, washed, and finally dehydrated through an ethanol series. For RNA in situ hybridization, sense or antisense *ZmACS* or *ZmACO* RNA was denatured at 80ºC, added to hybridization solution (0.3 M NaCl, 10 mm Tris-HCl, pH 6.8, 10 mm NaHPO4, 5 mm EDTA, 50% formamide, 10% dextran sulphate, 1 X Denhardts, 1 mg/ml tRNA), and applied to the slide for overnight hybridization at 55ºC. The sections were washed, treated with RNase, blocked (using 1.0% Boehringer Block), and incubated with anti-DIG antibody. The sections were washed, covered with a NBT substrate solution, and developed in the dark for 1-3 days until the signal was visible.

Ethylene determination

Ethylene was measured from root sections of 5 day-old seedlings. Excised roots were allowed to recover for 2 hr prior to collecting ethylene. Roots were placed in glass vials with 0.5 ml of water to maintain hydration of the roots and the vials capped with a rubber septum. Following a 3-4 hour incubation, 0.9 ml of headspace was sampled from each vial and the ethylene content measured

using a 6850 series gas chromatography system (Hewlett-Packard, Palo Alto, CA) equipped with a HP Plot alumina-based capillary column (Agilent Technologies, Palo Alto, CA). Tissue fresh weight was measured for each sample. Three to four replicates were measured and the average and standard deviation reported.

ACC synthase mutants

A reverse genetic approach was used to screen for *Mu* insertions in *ZmACS* gene family members as described (28). Terminal-inverted-repeat (TIR)-PCR (32) was performed on pools of DNA collected from maize containing *Mu* using one primer from the target gene and one primer from the *Mu* TIR region. Candidate lines were screened by PCR using HotStarTaq (Qiagen). Of 13 candidate lines identified harboring a *Mu* insertion in one of the *ZmACS* genes, 5 were stably inherited in the first backcross to B73. The *Mu* insertion site was determined by sequencing across the *Mu/ZmACS* junction using the *Mu*-TIR primer. Four of the five insertion lines that stably inherited the transposon contained an insertion in *ZmACS2* and the fifth contained an insertion in *ZmACS6*. Candidate lines were backcrossed an additional four times to B73 and then self-pollinated to generate homozygous insertion lines.

Biometric measurements

To examine root growth in the presence of minimal physical resistance, wild-type, *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1* seedlings were germinated on filter paper in 0.5X Murashige and Skoog (MS) media at 25ºC in the presence or absence of 10 µM ACC. The lengths of the primary and seminal roots as well as the length of the mesocotyl, which represents the distance from the scutellum to the crown node were measured after 10 days of growth. Measurements from 15 seedlings were made and the average and standard deviation reported. To measure the rate of growth when physical resistance was minimal, 3 day-old wild-type and *Zmacs2-1/6-1* seedlings were fixed against vertically-positioned Whatman paper (with plastic backing to provide support) in a glass tank similar to the approach described by Whalen and Feldman (15). 0.5X MS media was added to the tank sufficient to maintain the paper in a moist state without submerging the growing roots. Growth of the roots along the moist paper could be observed and measured daily through the glass walls of the tank for the duration of the experiment without disturbing the seedlings. Measurements were taken daily from 23 seedlings and the average and standard deviation reported. To examine biomass accumulation in soil, wild-type, *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1* plants were grown in full sunlight for four weeks in 5 gallon pots (one plant per pot). Root biomass fresh and dry weight measurements were made from 12 wild-type and mutant plants and the average and standard deviation reported.

RESULTS

ZmACS and ZmACO gene family members are expressed in maize roots

Of the three members in the *ZmACS* gene family, *ZmACS2* and *ZmACS7* are closely related (95% amino acid identity) whereas the third gene (i.e., *ZmACS6*) is considerably more divergent (54% and 53% amino acid identity with *ZmACS2* and *ZmACS7*, respectively) (27). To determine which members are expressed in roots, primary roots of one week old maize seedlings were collected and divided into 0-2, 2-4, and 4-6 mm sections from the root tip as well as 6-10 mm and 10-20 mm from the root tip. qRT-PCR revealed that *ZmACS2*, *ZmACS6*, and *ZmACS7* are expressed in maize roots (Fig. 2.1.A). In contrast to β-tubulin, which was expressed at a relatively constant level from the root tip to at least 2 cm from the tip (Fig. 2.1.B), only a low level of *ZmACS2* expression was detected in the root cap and the meristematic regions of the root (1-1.5 x 106 transcripts/µg RNA within the apical 4 mm of the root tip) but the amount of *ZmACS2* mRNA increased as a function of the distance from the root tip within the first centimeter of the root (Fig. 2.1.A). Although low relative to the distal region of the root, expression of *ZmACS7* was detected within the root cap and the meristematic regions (2 x 108 transcripts/µg RNA within the apical 4 mm of the root tip) but like *ZmACS2*, its expression increased as a function of the distance from the root tip (Fig. 2.1.A). *ZmACS6* was expressed at a higher level than *ZmACS2* or *ZmACS7* in the root cap and in the meristematic regions of the

root, then declined in the cell elongation zone, but increased once again beyond this region (Fig. 2.1.A).

All four maize *ZmACO* genes, i.e., *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35*, are expressed in maize roots (Fig. 2.2). *ZmACO15* and *ZmACO35* were differentially expressed along the root in that each was expressed at a lower level in the first 6 mm from the root tip than in the distal region (Fig. 2.2). Expression from *ZmACO20* was similar but showed a peak of expression between 6-10 mm from the root tip that decreased in the distal region (Fig. 2.2). Expression from *ZmACO31* was relatively constant up to 1 cm from the root tip at a level similar to *ZmACO35* and increased moderately in the distal region (Fig. 2.2). These results demonstrate distinct patterns of expression for *ZmACS* and *ZmACO* gene family members.

ZmACS and ZmACO gene family members exhibit cell specific patterns of expression in maize roots

To examine in greater detail the tissue specificity of *ZmACS* and *ZmACO* expression, in situ RNA localization was performed on primary roots of one week old seedlings. Expression of *ZmACS6* was detected in the peripheral cell layer of the root cap (Fig. 2.3.A-C) and in the outer cell layers of the cortex proximal to the quiescent center (QC) (Fig. 2.3.E). *ZmACS6* expression was not detected in

the calyptrogen of the root cap or in the columella (central column of cells of the root cap) (Fig. 2.3.B). No expression was detected in the QC (Fig. 2.3.A).

Because of the high degree of homology between *ZmACS2* and *ZmACS7*, the in situ RNA localization analysis could not discriminate between these two members and therefore the analysis represents the combined expression of *ZmACS2* and *ZmACS7* (i.e., *ZmACS2/7*). *ZmACS2/7* expression was detected in inner cortical cells adjacent to the vascular cylinder in the elongation zone (Fig. 2.3.F). No expression was observed in the outer cortical cells in the elongation zone or in the outer cortex proximal to the QC as had been observed with *ZmACS6* (Fig. 2.3.E). Like *ZmACS6*, no expression was detected in the QC (Fig. 2.3.F and I). Little expression of *ZmACS2/7* was detected in the root cap (Fig. 2.3.F) although prolonged incubation revealed a low level of expression in the tip of the root cap (Fig. 2.3.G).

Although *ZmACS* expression may serve as an indicator of where ACC is synthesized, ACC oxidase is responsible for the actual production of ethylene. Therefore, *ZmACO* expression likely serves as a better indicator of where ethylene is produced in the maize root. The four members of the *ZmACO* gene family form two subgroups based on sequence identity where *ZmACO20* and *ZmACO35* form one subgroup and *ZmACO15* and *ZmACO31* form a second subgroup (27). Because of the high degree of similarity within each subgroup, the *in situ* RNA localization analysis represents the combined expression of

ZmACO20 and *ZmACO35* (i.e., *ZmACO20/35*) and the combined expression of *ZmACO15* and *ZmACO31* (i.e., *ZmACO15/31*). Expression of *ZmACO15/31* was detected predominantly in the root cap (Fig. 2.3.J) where it was expressed strongly in all cells except for the calyptrogen (Fig. 2.3.L). No signal was detected in the root cap when sections were probed with *ZmACO15/31* sense RNA (Fig. 2.3.K). Analysis of cross sections at different positions within the root cap suggested that *ZmACO15/31* was expressed throughout cells of the root tip but was concentrated in the columella (Fig. 2.3.M-P). No expression was detected in the QC or the cortex (Fig. 2.3.J and L). However, *ZmACO15/31* expression was detected in the PSE prior to its enucleation and partial autolysis (Fig.2. 3.Q and V-Y). Expression of *ZmACO15/31* was not induced simultaneously in all PSE cells (Fig. 2.3.R and S) but sequential induction in all PSE was observed within 1-2 cells of the cell file. Induction of *ZmACO15/31* expression occurred prior to the appearance of the metaphloem sieve element (MSE) (Fig. 2.3.V-Y) and *ZmACO15/31* mRNA was detected in all PSE following enucleation when all MSE are evident (Fig. 2.3.Y-Aa). *ZmACO15/31* expression also appeared prior to the elongation and enucleation of the metaxylem (Fig. 2.3.V-Aa). No signal was detected in PSE cells in sections probed with *ZmACO15/31* sense RNA (Fig. 2.3.T and Bb). Expression of *ZmACO15/31* was not observed to be localized specifically in PSE cells in the mature region of the root (data not shown), data suggesting that *ZmACO15/31* expression in PSE cells was transient and corresponded to the period of the development of the PSE. No signal was

detected in the MSE, indicating highly cell specific expression of *ZmACO15/31* within the phloem in addition to its temporal regulation in the phloem.

Like *ZmACO15/31*, expression of *ZmACO20/35* was detected predominantly in the root cap where it was concentrated largely in the columella (Fig. 2.4.A). No signal was detected in the root cap when sections were probed with *ZmACO20/35* sense RNA (Fig. 2.4.B). Following long development of the signal, *ZmACO20/35* expression could be detected in most mature cells of the root cap (Fig. 2.4.C-D). Analysis of cross sections at different positions within the root cap suggested that *ZmACO20/35* was expressed to the highest extent in the columella (Fig. 2.4.E-F). No expression of *ZmACO20/35* was detected in the calyptrogen (Fig. 2.4.A, D, and G), the QC (Fig. 2.4.A, C, and I), or the cortex (Fig. 2.4.A and C). No signal was detected in the calyptrogen or the QC in sections probed with *ZmACO20/35* sense RNA (Fig. 2.4.H and J). Expression of *ZmACO20/35* was detected in the CC adjacent to the MSE (Fig. 2.4.K, M-Q). Expression of *ZmACO20/35* appeared following enucleation of the PSE but prior to the elongation and enucleation of the MSE and prior to the elongation and enucleation of the metaxylem (Fig. 2.4.R-U). Expression of *ZmACO20/35* appeared to be induced at each phloem element (Fig. 2.4.M-N) within one or both CC (Fig. 2.4.R-U) and was not limited to any one side of the root (Fig. 2.4.M). No signal was detected in CC in sections probed with *ZmACO20/35* sense RNA (Fig. 2.4.L).

Loss of ZmACS6 or ZmACS2 expression reduces ethylene production within the root tip

Zmacs2 or *Zmacs6* insertion mutants were isolated previously by screening for the presence of a *Mu* transposable element into gene (28). *Zmacs2-1* contains an insertion in the third exon whereas *Zmacs2-2*, *Zmacs2-3*, and *Zmacs2-4* contain insertions in the fourth exon at unique positions. *Zmacs6-1* contains an insertion in the second intron near the 3' splice site. qRT-PCR demonstrated that the insertion of *Mu* reduced foliar expression of *ZmACS2* to a non-detectable level and reduced *ZmACS6* expression to 0.01% of the wild-type level in the oldest leaves and 4-5% in the youngest leaves of mature plants (28). Residual *ZmACS6* expression may have resulted from the removal of *Mu* through splicing of the second intron in which the transposon resides. Foliar ethylene production in the *Zmacs2* and *Zmacs6* mutants was reduced to 55% and 10%, respectively, of wild-type levels (28).

To determine whether ethylene production was reduced in *Zmacs2* and *Zmacs6* roots, ethylene was measured from the apical 4 mm of primary roots of 5 day-old wild-type and mutant seedlings. Ethylene evolution from *Zmacs2-1*, *Zmacs2-2*, *Zmacs2-3*, and *Zmacs2-4* roots was 59%, 58%, 52%, and 49%, respectively, of wild-type levels (Fig. 2.5). Ethylene evolution from *Zmacs6-1* roots was 28% of that from wild-type roots (Fig.2. 5). In roots of *Zmacs2-1/6-1* double mutant plants, the level of ethylene production was reduced to 15% of the

wild type (Fig. 2.5). The greater reduction in ethylene production in *Zmacs6-1* roots compared to *Zmacs2-1* roots is consistent with the higher level of *ZmACS6* transcript accumulation in wild-type roots (Fig. 2.1.A). These data demonstrate that *ZmACS6* is largely responsible for ethylene production in the root tip with a small contribution provided by *ZmACS2*. The approximately 2-fold reduction in ethylene evolution in *Zmacs2* mutants was larger than perhaps expected given that the steady state level of *ZmACS2* mRNA was three orders of magnitude lower than for *ZmACS6* within the apical 4 mm of the root. However, in addition to the level of its transcript, the level of *ZmACS2* activity would be determined by the translational efficiency of the mRNA, the stability of the protein, and the activity of the enzyme. Moreover, transport of ACC from another region of the root cannot be ruled out. Therefore, the seemingly large reduction in ethylene production in *Zmacs2* roots may be a result of reduced ACC production in the distal region of the root where *ZmACS2* expression is relatively high (Fig. 2.1.A).

Ethylene controls root cap development and xylem cell elongation

To determine whether a reduction in the ability to synthesize ethylene affected maize root growth or development, the root cap and the division and elongation zones of roots from 5 day-old wild-type and *Zmacs2-1/6-1* seedlings were examined. The *Zmacs2-1/6-1* double mutant was used as it exhibited the greatest reduction in ethylene production and therefore would be most likely to

reveal any role that ethylene may have on root growth or development. Roots were fixed and embedded in resin prior to sectioning and staining. No gross alteration in root development was observed in either mutant (data not shown). However, detailed analysis revealed specific changes in the root cap and in the rate of cell elongation in the root proper.

The area of the root cap in *Zmacs2-1/6-1* roots, as determined from median longitudinal sections, was significantly smaller than wild type ($P<0.001$, Table 2.1). The decrease in the size of the root cap was accompanied by a significant increase in cell number ($P<0.001$) with a significantly smaller maximum cell size ($P<0.001$). *Zmacs2-1/6-1* roots exhibited a 2-fold increase in the number of peripheral cells, i.e., those that are in the process of sloughing off. Complementing the mutation with 10 µM ACC partially restored the size of the root cap, and fully restored the root cap cell number and the number of peripheral cells to those of wild-type roots (Table 2.1). These data suggest that ethylene regulates root cap size and cell number.

An increase in vacuolation was observed in cells of the calyptrogen of the root cap and in cells of the QC of *Zmacs2-1/6-1* roots relative to the wild type (Table 2.1). 26.0% of the cell area within the calyptrogen of *Zmacs2-1/6-1* roots was vacuolated compared to 15.3% for the wild type, a difference that was significant ($P<0.001$). Exogenous treatment of *Zmacs2-1/6-1* roots with 10 µM ACC reduced the vacuolation to a level that was not significantly different from

the wild type (P=0.314) but was significantly different from the mutant (P<0.001). The increase in vacuolation in the QC of *Zmacs2-1/6-1* roots was not significantly different from the wild type (P=0.152). Treatment of *Zmacs2-1/6-1* roots with 10 µM ACC reduced the vacuolation to a level that was not significantly different from the wild type (P=0.252) but was significantly different from the mutant (P<0.05). These observations suggest that ethylene may be involved in controlling the meristematic anatomy of cells in the calyptrogen, consistent with the observed increase in cell number of the mutant root cap.

A significant increase (P<0.001) in the longitudinal axis in cells of the metaxylem in *Zmacs2-1/6-1* roots was observed without a significant increase (P=0.139) in the transverse axis relative to the wild type (Table 2.1). The increase was observed beginning with the cells proximal to the QC. Treatment of *Zmacs2-1/6-1* seedlings with 10 µM ACC partially reversed the mutant phenotype such that the longitudinal axis of metaxylem cells in the ACC-treated mutant roots was significantly different from the untreated mutant (P<0.001) and approached that of the wild type although the difference between them remained significantly different (P<0.05). The transverse axis of metaxylem cells in the ACC-treated mutant roots was not significantly different from the wild type (P=0.766) but was significantly less than the untreated mutant (P<0.05). A significant increase in the longitudinal axis of expanding mutant cortical (P<0.001) and epidermal (P<0.001) cells relative to the wild type was also observed (Table 2.1). Treatment with 10 µM ACC partially or fully reversed these mutant

phenotypes. The transverse axis of epidermal cells in mutant roots was also significantly larger than in wild-type roots and was reversed by treatment with 10 µM ACC. Although the transverse axis of cortical cells in mutant roots was significantly larger than the wild type, the difference was small and was not reversed by treatment with 10 µM ACC. Similar results were observed for fully-expanded cells (Table 2.2). Fully-expanded cortical and epidermal cells were substantially longer in *Zmacs2-1/6-1* roots relative to the wild type cells ($P<0.001$), a phenotype largely reversed by treatment with 10 µM ACC (Table 2.2). These data suggest that the increase in root cell length in the mutant was due to reduced ethylene production and that loss of *ACS* expression affects the early expansion of the cells and their mature size.

ZmACS6 regulates root growth

To determine whether root growth was affected by the reduction in ethylene evolution observed in the *Zmacs* mutants, the rate of unimpeded growth of roots from wild-type and *Zmacs2-1/6-1* seedlings was examined. Three day-old seedlings with similar root length were fixed against vertically-positioned Whatman paper in a glass tank containing 0.5X MS media sufficient to maintain the paper in a moist state without submerging the growing roots. The paper provided moisture without imposing substantial physical resistance and the growth of the primary root could be measured daily through the glass for the

duration of the experiment without disturbing the seedlings. During the first two days, a similar rate of root growth was observed between wild-type and *Zmacs2-1/6-1* seedlings (Fig. 2.6). Subsequent to this, *Zmacs2-1/6-1* roots grew at a significantly higher rate ($P<0.005$, paired t-test) than did the wild type and continued to do so for the duration of the experiment (Fig. 2.6). The increased rate of elongation of *Zmacs2-1/6-1* roots was consistent with the increased cell length (Tables 2.1 and 2.2).

To determine the relative contributions of *ZmACS2* and *ZmACS6* to root growth, the growth of *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1* roots was compared to those of wild-type plants in the presence of minimal physical resistance. The lengths of the primary roots emerging from the embryo, the seminal roots emerging from the scutellar node, and the mesocotyl, which represents the distance from the scutellum to the first node, were measured. When grown on moist filter paper for 5 days, the primary roots of *Zmacs6-1* seedlings were significantly longer than wild-type roots as were those of the *Zmacs2-1/6-1* mutant (Fig. 2.7.A). Ethylene evolution was lower in *Zmacs6-1* and *Zmacs2-1/6-1* roots compared to wild-type roots (Fig. 2.7.D). In contrast, the length of *Zmacs2-1* primary roots was similar to wild-type roots (Fig. 2.7.A) as was ethylene evolution (Fig. 2.7.D). Application of exogenous ACC disproportionately reduced the growth of *Zmacs6-1* and *Zmacs2-1/6-1* primary roots relative to the wild type, resulting in little to no difference in growth between the mutant and wild-type roots (Fig. 2.7.A). The application of exogenous ACC

resulted in a substantial increase in ethylene evolution in the roots of wild type and mutant seedlings with little difference in the relative level observed among them (Fig. 2.7.D), which correlated with a similar primary root length among mutant and wild-type seedlings.

As observed with primary roots, seminal roots from the *Zmacs6-1* single mutant and the *Zmacs2-1/6-1* double mutant were longer than the wild type (Fig. 2.7.B). Seminal roots from the *Zmacs2-1* mutant showed a smaller, but significant increase in length relative to the wild type (Fig. 2.7.B). Application of exogenous ACC disproportionately reduced the growth of the seminal roots from the mutants, resulting in little to no difference in growth between the mutant and wild-type roots (Fig. 2.7.B). In contrast to the significant increase in primary and seminal root growth, the length of the mesocotyl in the *Zmacs2-1* or the *Zmacs6-1* mutants was not significantly different from the wild type (Fig. 2.7.C), however, the length of the mesocotyl in the *Zmacs2-1/6-1* mutant was significantly longer than the wild type. Application of exogenous ACC reduced slightly the growth of the mesocotyl in the wild type and *Zmacs2-1* mutant but had little effect on the mesocotyls of *Zmacs6-1* or *Zmacs2-1/6-1* seedlings (Fig. 2.7.C). These results suggest that *ZmACS6* functions to limit root growth in the presence of minimal physical resistance.

As ethylene has been implicated to promote root growth in soil which presents mechanical impedance (9, 11), the growth of *Zmacs2-1*, *Zmacs6-1*, and

Zmacs2-1/6-1 roots was compared to those of wild-type plants in soil. Plants were grown for four weeks in soil to simulate normal growth conditions, following which, the fresh and dry weight of each root mass were determined. Growth of wild-type in soil resulted in increased ethylene evolution (compare Table 2.3 to Fig. 2.7.D). As in the case of minimal physical resistance, ethylene evolution was highest in wild-type and *Zmacs2-1* roots and lowest in *Zmacs6-1* and *Zmacs2-1/6-1* roots. The root dry weight biomass of *Zmacs6-1* plants was significantly lower than the wild type (i.e., 80.4% of wild type, $P<0.01$). Similarly, the root dry weight biomass of *Zmacs2-1/6-1* plants was significantly lower than wild type (i.e., 78.4% of wild type, $P<0.005$). Similar effects were observed in the fresh weight of the roots. The reduction in root biomass in *Zmacs6-1* and *Zmacs2-1/6-1* plants correlated with reduced growth of the primary root in 10 day-old seedlings relative to the wild type (i.e., 71.4% $P<0.01$ and 79.1% of wild type, $P<0.05$, respectively). In contrast, the root dry weight biomass of *Zmacs2-1* plants was not significantly different from the wild type (i.e., 98.3% of wild type, $P = 0.801$) correlating with no significant change in the growth of the primary root in *Zmacs2-1* seedlings relative to the wild type (i.e., 97.8% of wild type, $P = 0.814$). These data suggest that *ZmACS6* serves to maintain root biomass during normal growth in soil.

To determine if expression of the ethylene biosynthetic and perception machinery is altered in response to growth in soil, RT-PCR analysis of roots grown in the absence of most physical resistance was performed and compared

to that of roots grown in soil. Expression from *ZmACS6* was substantially higher in soil-grown roots relative to those grown in the absence of most physical resistance (Fig. 2.8). No expression from *ZmACS2* or *ZmACS7* was detected, in good agreement with the data of Fig. 2.1.A that showed their poor expression in the root tip. Expression from *ZmACO20*, and to lesser extent *ZmACO35*, was also higher in soil-grown roots relative to roots grown in the absence of most physical resistance (Fig. 2.8). Expression from *ZmACO15* and *ZmACO31* was lower in soil-grown roots relative to growth in the absence of most physical resistance (Fig. 2.8). *ZmERS1* expression was higher as was expression from *ZmERF1* following growth in soil relative to growth in the absence of most physical resistance whereas little change was detected in *ZmETR2* expression (Fig. 2.8). No change in expression from *ZmXET* or *eIF4A*, which was used as a control, was observed in soil-grown roots relative to growth in the absence of most physical resistance (Fig. 2.8). These data show changes in expression of specific members of the ethylene biosynthetic and perception machinery gene families in response to growth in soil that are consistent with changes in ethylene production and responses.

DISCUSSION

In this study, it is shown that cell specific expression of *ZmACS* and *ZmACO* gene family members in maize roots and that *ZmACS6* contributes

substantially to the regulation of root growth in response to soil conditions. qRT-PCR revealed that *ZmACS6* was expressed in the root tip to a substantially higher level than was *ZmACS2* or *ZmACS7*. *In situ* RNA localization analysis confirmed this finding in that *ZmACS6* transcripts could be readily detected in the peripheral cells of the root cap. Because of their high degree of homology, the *in situ* hybridization could not distinguish between *ZmACS2* and *ZmACS7*. However, only a low level of *ZmACS2/7* transcript could be detected in the root cap and only after prolonged development of the signal. Expression of *ZmACS6*, *ZmACS2*, and *ZmACS7*, as measured by qRT-PCR, increased in the distal region of the root proper. Although no expression was observed in the QC, *ZmACS6* expression was detected in the outer cells of the cortex whereas expression of *ZmACS2/7* was detected in the inner cells of the cortex, observations supporting the qRT-PCR results. The region of the root in which cortical expression of *ZmACS6* and *ZmACS2/7* was observed corresponded to the zones of cell division, differentiation, and elongation. Uniform expression of the *ZmACS* genes, as determined by *in situ* hybridization, occurred in the mature portion of the root (data not shown), data correlating with the qRT-PCR results which indicated that all three *ZmACS* gene family members are expressed at relatively high levels in this region of the root. Extensive screening for *ZmACS* genes identified only three members for this gene family (27) although, the existence of other members cannot be formally ruled out. However, the observation that loss of *ZmACS2* and *ZmACS6* expression results in loss of

more than 85% of ethylene production in roots of the *Zmacs2/Zmacs6* double mutant suggests that either *ZmACS7* is responsible for the remaining 15% of ethylene production or that any additional unidentified *ZmACS* gene family members make a relatively minor contribution to the total production of ethylene.

The members of the *ZmACO15/31*subgroup were expressed in the root cap and their expression increased across the distal 2 cm of the root. The members of the *ZmACO20/35* subgroup were expressed in the root cap to a lower level than were the members of the *ZmACO15/31* subgroup but their expression also increased across the distal 2 cm of the root. The qRT-PCR results were confirmed by in situ RNA localization analysis in which expression of the *ZmACO15/31* subgroup was readily detected in the columella and peripheral cells of the root tip whereas expression of the *ZmACO20/35* subgroup required longer development of the signal to detect and appeared to be highest in the columella. No expression from either *ZmACO* subgroup was detected in the calyptrogen. In the root proper, *ZmACO* expression was limited to the phloem. The *ZmACO15/31* subgroup was expressed specifically in the PSE prior to its enucleation and prior to the enucleation of the MSE. In contrast, the *ZmACO20/35* subgroup was expressed specifically in the CC associated with the MSE. *ZmACO20/35* expression was observed following enucleation of the PSE but before enucleation and elongation of the MSE.

These findings demonstrate coordinate expression of *ZmACS* and *ZmACO* genes in the root cap, suggesting that the root cap is competent to generate ethylene. Ethylene has been shown to influence gravitropism (7, 8, 33, 34) perhaps by influencing polar or lateral auxin transport (8, 35, 36) or by positively regulating auxin synthesis in the root cap as supported by the isolation of two weak ethylene insensitive mutants, *wei2* and *wei7* that encode enzymes involved in Trp synthesis (37). The root cap also serves as a sensor of its environment, e.g., soil density (7). The observation that the root cap expresses the biosynthetic enzymes necessary for ethylene production is consistent with the role that the root cap plays in these response programs. Although no substantial difference in gravitropism was observed in the *Zmacs* mutants, the residual level of ethylene produced in the mutants may be sufficient to maintain a wild-type response. A complete ethylene null mutant would be needed to demonstrate such a role. In contrast to the root cap, expression from *ZmACS* and *ZmACO* genes appeared to be spatially separate in the zones of cell division and expansion of the root proper, suggesting that ACC may be transported from the site of its generation to those cells expressing ACO. The ability of ACC to be taken up by roots and converted to ethylene is demonstrated in the ACC feeding experiments in Fig. 2.7.D where elevated ethylene production was observed.

These findings share similarities and differences with what has been observed in deepwater rice and Arabidopsis. Expression of Arabidopsis *ACS8* was observed in the root cap similar to the expression pattern observed for

ZmACS6 in that *ACS8* expression was confined to the outermost two cell layers (21). Seven of the nine members of the Arabidopsis *ACS* gene family are expressed in root vascular tissue, primarily in the protoxylem but also in the protophloem in the case of *ACS2* and *ACS4* (21). Expression of *OS-ACS5*, a submergence-induced member of the rice *ACS* gene family (22, 23), was detected in the elongation zone of lateral roots of air-grown plants (24), similar to the expression detected for *ZmACS6* and *ZmACS2/7*. Expression of *OS-ACS5* was also detected in the vascular tissues (both phloem and xylem) of stems and young leaves of air-grown plants (24), however, detailed expression analysis of *OS-ACS5* in roots was not reported. ACC oxidase expression analysis has not been reported for either Arabidopsis or rice so it is unknown whether the root cap and phloem-specific expression observed for the *ZmACO15/31* and *ZmACO20/35* subgroups is conserved in other species.

As the product of the reaction catalyzed by ACC oxidase is ethylene, the location of *ACO* expression is likely the best indicator of where ethylene is actually produced. Expression of *ZmACS* and *ZmACO* in the root proper appears to occur in largely spatially separate cell types where *ZmACS7* expression in the inner cortex is at least one cell layer away from the phloem-specific expression of *ZmACO*. The phloem is composed of sieve elements and the CC in addition to several other cell types (38). The primary phloem, initiated in the embryo, is classified into the protophloem and metaphloem where the protophloem differentiates prior to the metaphloem. The protophloem pole originates from a

single phloem mother cell which produces the sieve elements and the CC. Differentiation of the sieve element follows completion of the cell divisions and involves breakdown of the nucleus and selective autophagy that includes loss of ribosomes, Golgi bodies, microtubules, and microfilaments (38). Degeneration occurs rapidly during the final stages of sieve element differentiation. Although mature sieve elements retain a fully functional plasma membrane, the tonoplast breaks down during the final stages of sieve element differentiation so that mature sieve elements lack vacuoles. The function of the enucleate sieve elements is maintained with the support of the CC through transfer of material, e.g., solutes and RNA, via their extensive plasmodesmatal connections. The CC remain nucleate and retain a dense cytoplasm at maturity (38). Protein translocation can also occur and some proteins present in sieve elements are synthesized in the CC (39, 40).

The expression of the *ZmACO15/31* subgroup in the PSE but not the MSE represents a surprisingly degree of specificity and could suggest that *ZmACO* expression is not required for MSE development. However, the observation that *ZmACO20/35* subgroup is expressed in the CC that are associated with the MSE may provide a means by which ACC oxidase could be transported to the MSE. Although PSE are shorter lived than MSE, the expression of *ACO* may not be responsible for the immediate cessation of PSE function as it occurs prior to the final stages of PSE differentiation but a role in PSE development can not be ruled out. It is also possible that *ACO* mRNA and/or protein made in the CC could be

transported to the sieve elements for translocation through the sieve tube to other regions of the root. The CC are thought to be unnecessary for the differentiation of the protophloem (39). Therefore, if the ethylene produced in the CC is involved in the differentiation of the protophloem, it may be limited to regulating the onset or rate of protophloem differentiation. Because of the proximity of the protophloem to the metaxylem, it is also possible that the *ACO* produced in the PSE and the CC is involved in the differentiation of the metaxylem through diffusion of ethylene from the phloem. The role that *ZmACO15/31* may play in the development of the PSE or the role that *ZmACO20/35* may play in the development of the CC will require mutants in these genes.

In order for the *ACO* expressed in the PSE and the CC to synthesize ethylene, ACC must be present. As *ZmACS* expression appears to be restricted to the root cortex within the root proper, transport of ACC to the phloem would be a necessary prerequisite for the generation of ethylene. Transport of ACC to and from roots has been reported. ACC synthesized in anaerobic tomato roots is transported through the xylem to the shoot where it is converted to ethylene (41). Transport of ACC in tomato subjected to flooding has been suggested to be important in root-shoot signaling (42). Transport of ACC from roots to shoots was also shown in sunflower and Cleopatra mandarin seedlings (43, 44). ACC transport from leaves to roots was observed in tomato, suggesting that ACC can also be transported through the phloem (45). Thus, ACC may be transported to

cells of the phloem from the root cortex, the root cap, or the basal portion of the maize root where *ZmACS* expression occurs. ACC transport through the phloem might enable ACC synthesized in distal regions of the root to reach the cells of the developing phloem where ACO is expressed.

No gross developmental defects were observed in the roots of the *Zmacs* mutants, suggesting either that ethylene is not essential for root development or that the residual level of ACC generated in the mutants is sufficient. A complete ACS null mutant would be needed to distinguish between these two possibilities. A role for ethylene in maize root growth, however, was suggested by the analysis of *Zmacs* mutants. The 85% reduction in ethylene production in *Zmacs2-1/6-1* roots resulted in a smaller root cap composed of more cells of smaller size than the wild type (Table 2.1). Consistent with the increase in cell number, the root cap of *Zmacs* mutant roots contained more peripheral cells, i.e., those in the process of sloughing off. The root cap size, maximum cell size, cell number, and number of peripheral cells of the mutant could be partially or fully reversed by exogenous ACC, demonstrating that this mutant phenotype resulted from the reduction in ACS activity. The increase in the number of root cap cells is consistent with the increased vacuolation in cells of the calyptrogen in the mutant, suggesting a change in their meristematic anatomy. A reduced rate of root cap cell death may also contribute to the greater number of root cap cells, especially peripheral cells, in the mutant.

An increase in the longitudinal axis of metaxylem, cortical, and epidermal cells was also observed in *Zmacs* mutant roots, a phenotype that could be reversed by exogenous ACC (Tables 2.1and 2.2). These results suggest that ethylene may serve to regulate the maturation of these cell types. Ethylene has been shown to regulate cell elongation in roots, typically by repressing cell elongation while promoting radial expansion although exceptions have been observed (15, 17, 46). The effect of ethylene on root cell elongation is auxin-dependent (47-49). That ethylene may also regulate events in the meristem has received support from the observed reduction in mitosis and DNA synthesis in the root apex of pea (50). Moreover, inhibition of ethylene biosynthesis by aminoethoxyvinylglycine (AVG) resulted in the activation of cells in the QC and calyptrogen of maize roots (51), results that are consistent with the increase in root cap cell number and decrease in root cap cell size observed in *Zmacs* roots which could be reversed by exogenous ACC. In contrast, a role for ethylene in promoting cell division within the QC was observed in Arabidopsis roots (52), which may be due to structural differences between the root types or reflect a difference between the two species. Observations here support the notion that ethylene regulates the meristematic activity of the calyptrogen as well as the elongation of cells within the root proper. The increase in cell length during early cell expansion in *Zmacs* roots indicates that ethylene functions even during early cell growth. The mature cell size was similarly affected in that cortical and epidermal cells were considerably longer in *Zmacs* mutant roots than in wild-type

roots, a phenotype that could be complemented by exogenous ACC. These results are consistent with the faster growth of *Zmacs* roots in the presence of minimal physical resistance and suggest that ACS expression serves to regulate the early expansion of cells as well as the final size attained.

The higher level of *ZmACS6* expression in the root tip (Figs. 2.1 and 2.3) raised the question of whether this gene family member played a greater role in root growth than did *ZmACS2*. The analysis of *Zmacs6* and *Zmacs2* mutants revealed that loss of *ZmACS6* expression resulted in a greater reduction in ethylene evolution than did loss of *ZmACS2* expression, consistent with the qRT-PCR results that demonstrated a higher level of expression for *ZmACS6*. *Zmacs6* roots exhibited a greater rate of growth than did *Zmacs2* roots when growth was largely unimpeded (Fig. 2.7), an observation consistent with the observed increased cell length (Tables 2.1 and 2.2). The inhibition of maize root growth following treatment with exogenous ethylene and the stimulation of root growth by the pharmacological inhibition of ethylene signaling (15, 16) are also consistent with these observations.

In contrast, growth of *Zmacs6-1* roots in soil was significantly reduced relative to *Zmacs2-1* or wild-type roots, resulting in a significantly reduced root biomass (Table 2.3). This is consistent with our observation that *ZmACS6* is expressed to a substantially higher level in roots than is *ZmACS2* (Fig. 2.1), is responsible for the bulk of ethylene produced in roots (Fig. 2.5), and is expressed

in the root cap (Fig. 2.3) which functions as a sensor of soil density (7). Growth in soil resulted in increased expression from *ZmACS6* in the root tip (Fig. 2.7) but no detectable increase in *ZmACS2* or *ZmACS7* expression (data not shown). Expression from *ZmACO20* and *ZmACO35* (but not *ZmACO15* and *ZmACO31*) also increased as did expression from *ZmERS1* (but not *ZmETR2*) and *ZmERF1*, suggesting that growth in soil elicited specific changes in the ethylene biosynthetic and signaling machinery consistent with an increase in ethylene production (i.e., increased ZmACS expression which is considered rate limiting for ethylene production) and in the ethylene response (i.e., increased ZmERF1 expression). Interestingly, *ZmACO20* and *ZmACO35* share a high degree of similarity and constitute the *ZmACO20/35* subgroup (27), suggesting that members of this gene family may be similarly regulated. The increase in expression of *ZmACS6* in soil-grown, wild-type roots correlated with an increase in ethylene evolution that was not seen in *Zmacs6-1* roots (Table 2.3). Ethylene signaling has also been shown in tomato to be necessary for root growth in response to physical resistance (9, 11). Therefore, Results in this study suggest that ethylene plays a similar role in maize and that *ZmACS6* serves to regulate root growth in response to soil conditions.

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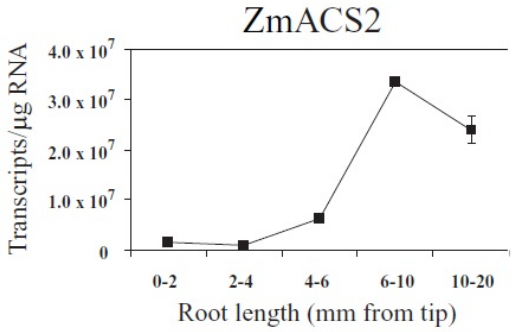
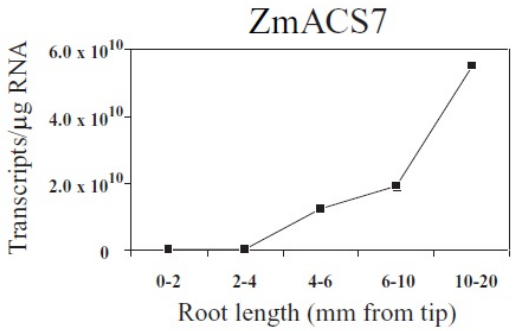
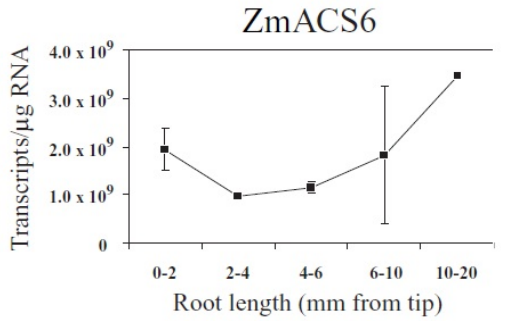
Figure 2.1. Quantitative expression analysis of *ZmACS* gene family members in maize roots. (A) Real time qRT-PCR analysis of expression of *ZmACS2*, *ZmACS7*, *and ZmACS6*. Real time qRT-PCR was performed on RNA isolated from 2 mm sections for the first 6 mm of the apical region of the root as well as 6-10 mm and 10-20 mm from the root tip. Transcript amounts are expressed in exponential form and plotted on a RNA basis. (B) Real time qRT-PCR analysis of β-tubulin gene expression. Each gene was analyzed a minimum of four times and the average and standard deviation are reported.

A


ZmACS2
Transcripts/µg RNA
4.0 x 10^7
3.0 x 10^7
2.0 x 10^7
1.0 x 10^7
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)


ZmACS7
Transcripts/µg RNA
6.0 x 10^10
4.0 x 10^10
2.0 x 10^10
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)


ZmACS6
Transcripts/µg RNA
4.0 x 10^9
3.0 x 10^9
2.0 x 10^9
1.0 x 10^9
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)

B

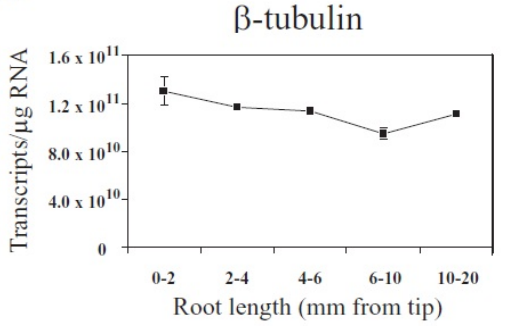
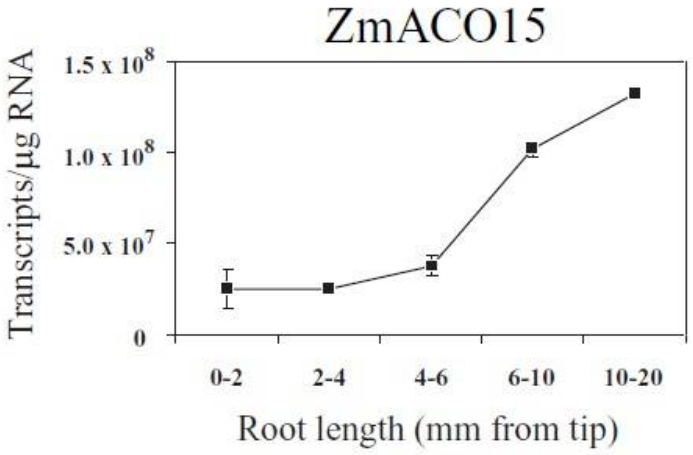
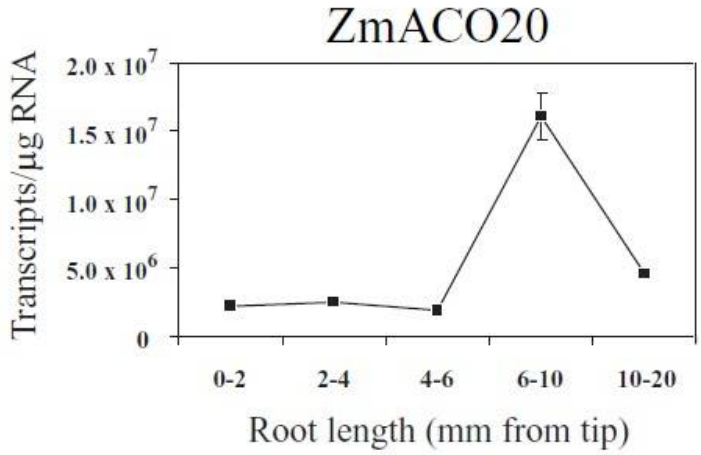
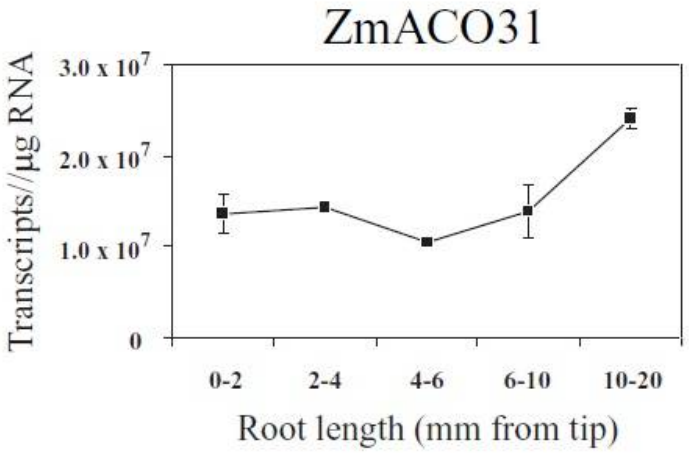

β-tubulin
Transcripts/µg RNA
1.6 x 10^11
1.2 x 10^11
8.0 x 10^10
4.0 x 10^10
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)

Figure 2.2. Quantitative expression analysis of *ZmACO* gene family members in maize roots. Real time qRT-PCR analysis of expression of *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35*. Real time qRT-PCR was performed on RNA isolated from 2 mm sections for the first 6 mm of the apical region of the root as well as 6-10 mm and 10-20 mm from the root tip. Transcript amounts during development are expressed in exponential form and plotted on a RNA basis. Each gene was analyzed a minimum of four times and the average and standard deviation are reported.


ZmACO15
Transcripts/µg RNA
1.5 x 10^8
1.0 x 10^8
5.0 x 10^7
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)


ZmACO20
Transcripts/µg RNA
2.0 x 10^7
1.5 x 10^7
1.0 x 10^7
5.0 x 10^6
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)


ZmACO31
Transcripts//µg RNA
3.0 x 10^7
2.0 x 10^7
1.0 x 10^7
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)

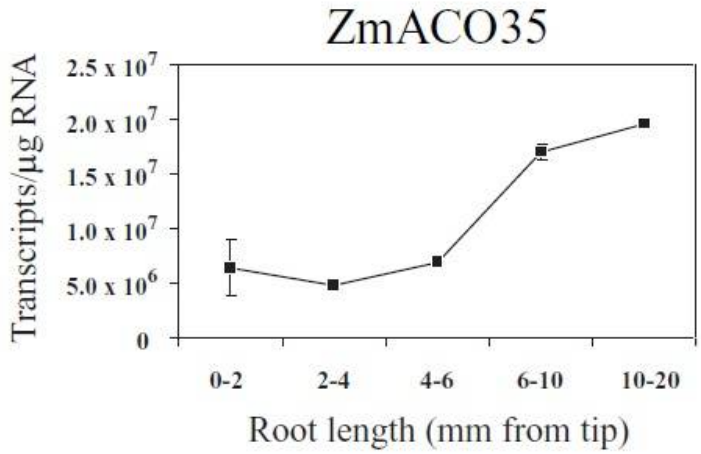

ZmACO35
Transcripts/µg RNA
2.5 x 10^7
2.0 x 10^7
1.5 x 10^7
1.0 x 10^7
5.0 x 10^6
0
0-2
2-4
4-6
6-10
10-20
Root length (mm from tip)

Figure 2.3. In situ localization of *ZmACS6*, *ZmACS2/7*, and *ZmACO15/31* mRNA in maize roots. Sections of root from one week old seedlings were hybridized with digoxygenin-labeled riboprobes representing *ZmACS6* (A-E), *ZmACS2/7* (F-I), or *ZmACO15/31* (J-Bb). Hybridization was detected as a blue precipitate by staining with NBT. Median longitudinal (A and B) and cross (C and E) sections probed with antisense *ZmACS6* RNA. (D) Cross section probed with sense *ZmACS6* RNA. The cross sections in (C-E) are indicated in (A). C, calyptrogen. S, sense probe control. Median longitudinal (F) and cross (G-I) sections probed with antisense *ZmACS2/7* RNA. The cross sections in (G-I) are indicated in (F). Median longitudinal (J, L, and U) and cross (M-S and V-Aa) sections probed with antisense *ZmACO15/31* RNA. Median longitudinal (K) and cross (T and Bb) sections probed with sense *ZmACO15/31* RNA. The cross sections in (O-Q) are indicated in (J). *x*, metaxylem; *mse*, metaphloem sieve element; *pse*, protophloem sieve element. Bar is equivalent to 100 µM except in V-Y where it is 50 µM.

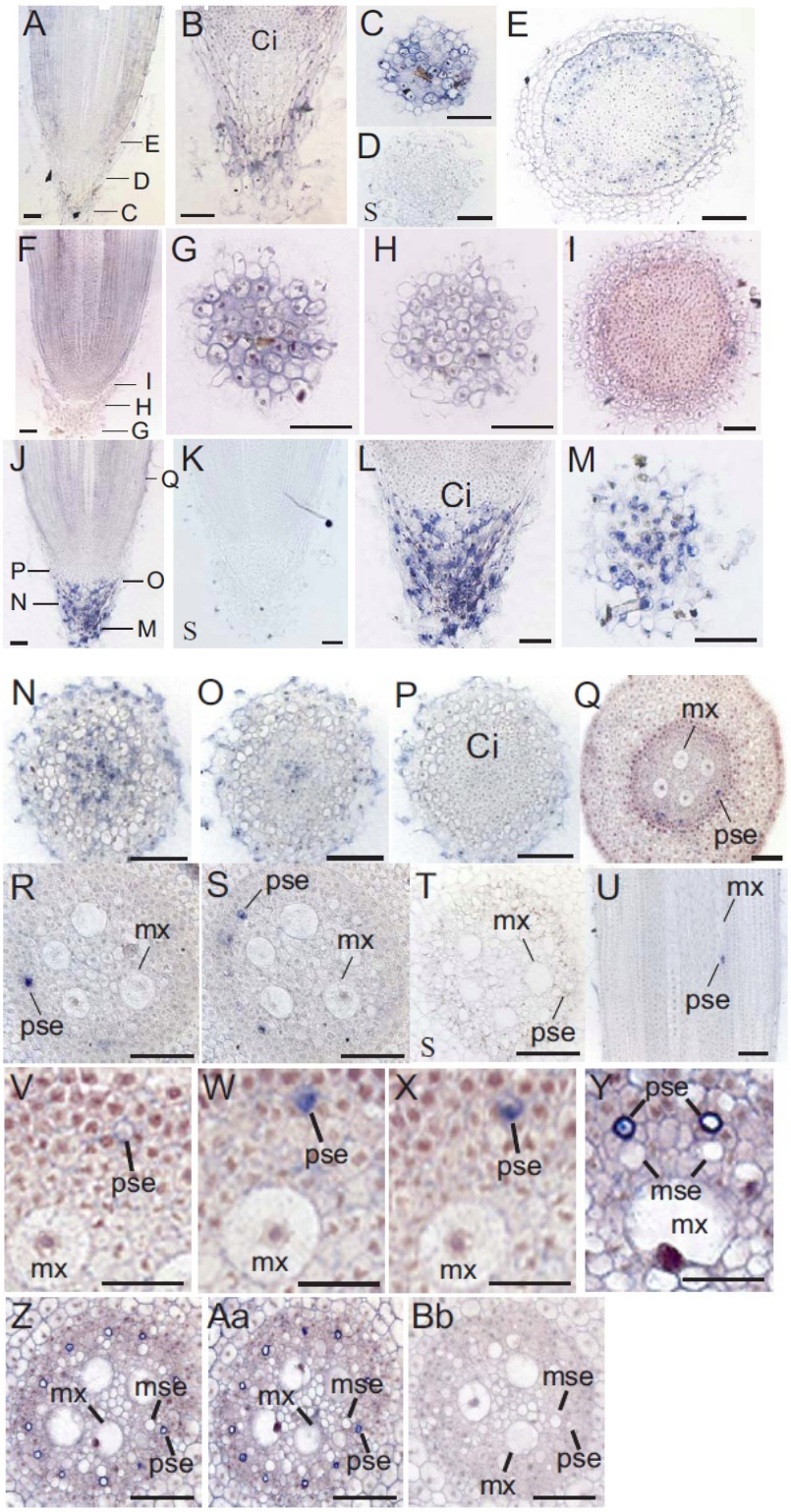

A
E
D
C
B
Ci
C
D
S
E
F
I
H
G
G
H
I
J
Q
P
O
N
M
K
S
L
Ci
M
N
O
P
Ci
Q
mx
pse
R
mx
pse
S
pse
mx
T
mx
pse
S
U
mx
pse
V
pse
mx
W
pse
mx
X
pse
mx
Y
pse
mse
mx
Z
mx
mse
pse
Aa
mx
mse
pse
Bb
mse
pse
mx

Figure 2.4. In situ localization of *ZmACO20/35* mRNA in maize roots. Sections of root from one week old seedlings were hybridized with digoxygenin-labeled *ZmACO20/35*. Hybridization was detected as a blue precipitate by staining with NBT. Median longitudinal (A, C, D, and O-Q) and cross (E-G, I, K, M, N, and R-U) sections probed with antisense *ZmACO20/35* RNA. Median longitudinal (B) and cross (H, J, and L) sections probed with sense *ZmACO20/35* RNA. The cross sections in (E-G, I, and K) are indicated in (C). *C*, calyptrogen; *x*, metaxylem; *mse*, metaphloem sieve element; *pse*, protophloem sieve element; *cc*, companion cell. *S*, sense probe control. Bar is equivalent to 100 µM.

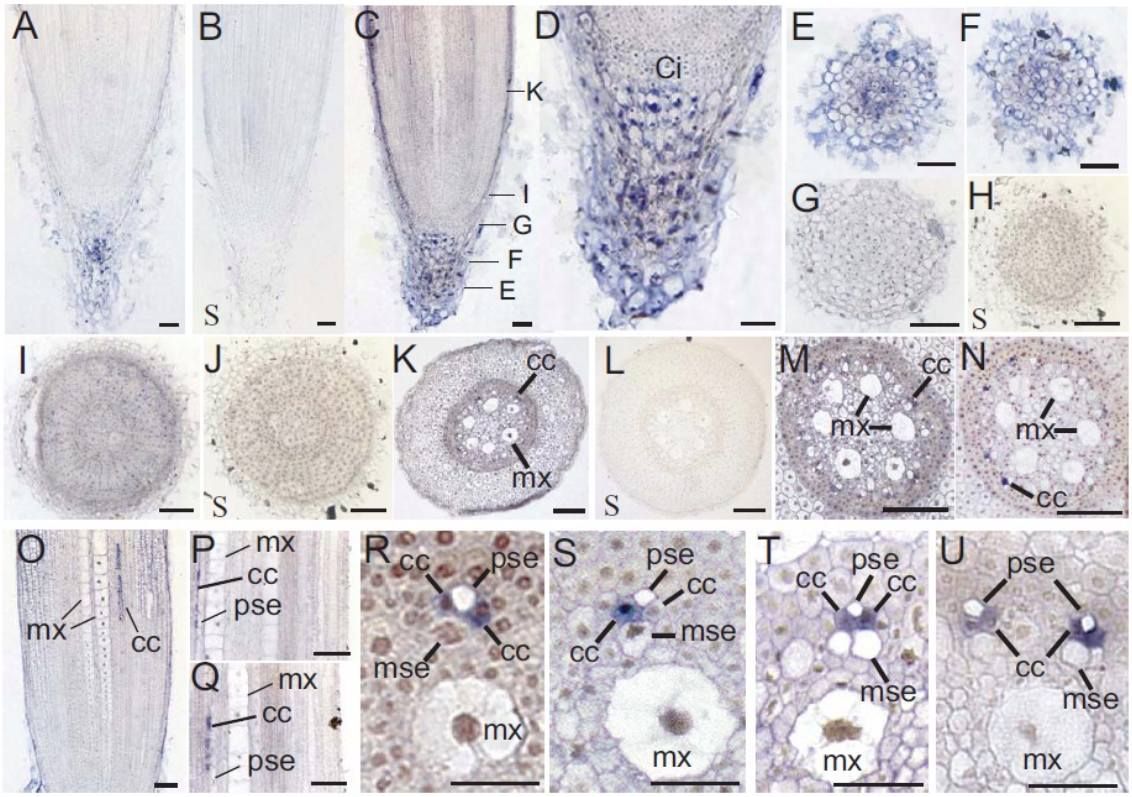

A
B
S
C
K
I
G
F
E
D
Ci
E
F
G
H
S
I
J
S
K
cc
mx
L
S
M
cc
mx
N
mx
cc
O
mx
cc
P
mx
cc
pse
Q
mx
cc
pse
R
cc
pse
mse
cc
mx
S
pse
cc
cc
mse
mx
T
cc
pse
cc
mse
mx
U
pse
cc
mse
mx

Figure 2.5. *ZmACS6* is responsible for the bulk of ethylene produced in maize roots. Ethylene production in roots of wild-type, *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1* plants. Three replicates were measured and the average and standard deviation reported.

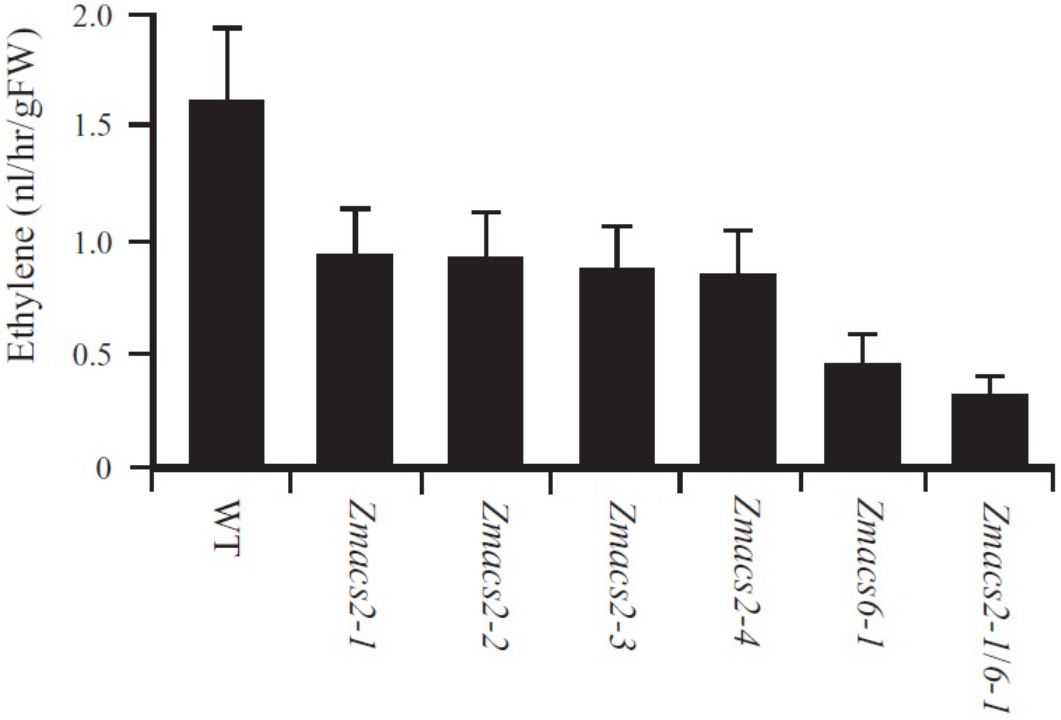

Ethylene (nl/hr/gFW)
2.0
1.5
1.0
0.5
0
WT
Zmacs2-1
Zmacs2-2
Zmacs2-3
Zmacs2-4
Zmacs6-1
Zmacs2-1/6-1

Figure 2.6. Reduction in ethylene evolution results in a higher rate of root elongation during unimpeded growth. Wild-type (open squares) and *Zmacs2-1/6-1* (filled squares) seedlings that were 3 days old were fixed against vertically-positioned Whatman paper in a glass tank. 0.5X MS media was added to the tank to maintain the paper in a moist state without submerging the growing roots. Growth of the primary roots (n=23) was measured beginning the day after transfer to the tank (4 days after imbibition) and daily thereafter. The average and standard deviation of the daily elongation of the primary root for each day are reported.

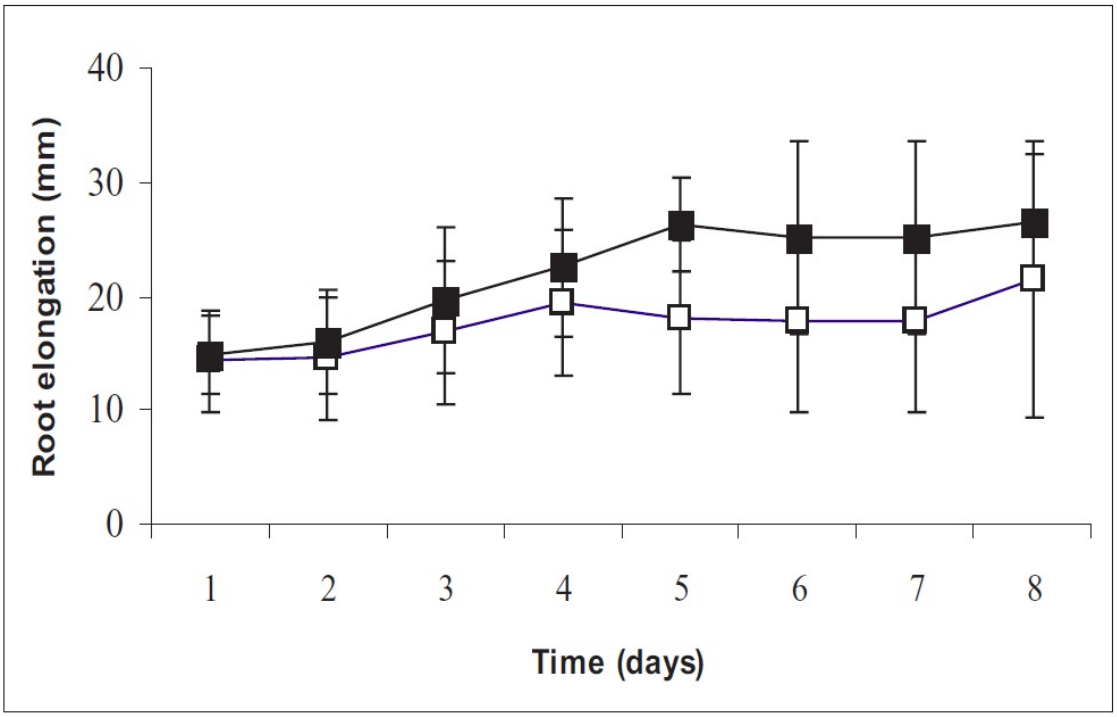

Root elongation (mm)
40
30
20
10
0
1
2
3
4
5
6
7
8
Time (days)

Figure 2.7. Regulation of root growth requires *ZmACS6*. Wild-type, *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1* seedlings were grown on filter paper with 0.5X MS (black bars) or 10 µM ACC (white bars). The lengths of (A) the primary roots emerging from the embryo, (B) the seminal roots emerging from the scutellar node, and (C) the mesocotyl, representing the distance from the scutellum to the first node, were measured following growth for 5 days. The average and standard deviation from 15 seedlings are reported. (D) Ethylene production in wild-type, *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1* roots grown on filter paper with 0.5X MS (black bars) or 10 µM ACC (white bars). Three replicates were measured and the average and standard deviation reported.

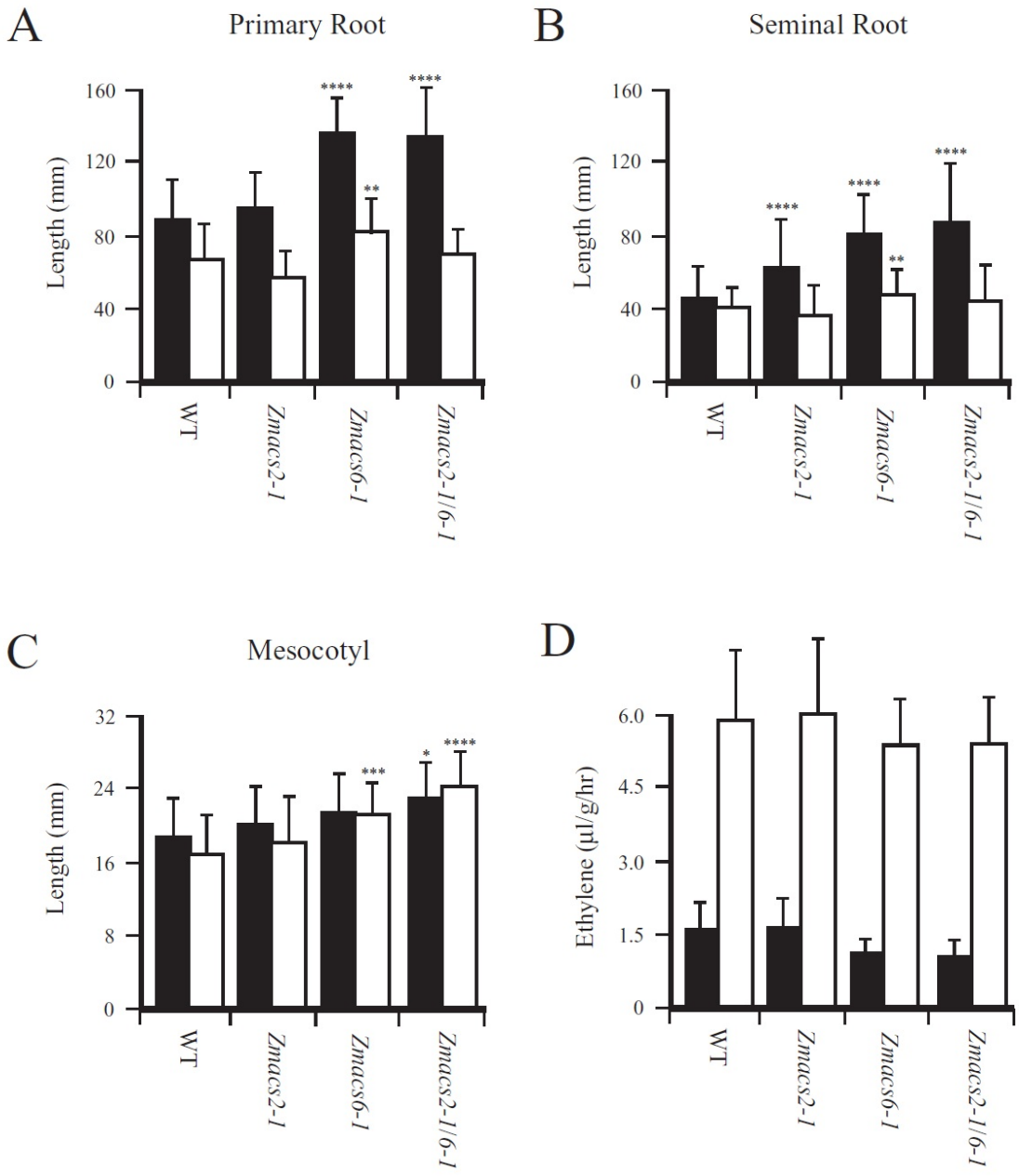

A
Primary Root
Length (mm)
160
120
80
40
0
****
**
****
WT
Zmacs2-1
Zmacs6-1
Zmacs2-1/6-1
B
Seminal Root
Length (mm)
160
120
80
40
0
****
**
****
****
WT
Zmacs2-1
Zmacs6-1
Zmacs2-1/6-1
C
Mesocotyl
Length (mm)
32
24
16
8
0
***
*
****
WT
Zmacs2-1
Zmacs6-1
Zmacs2-1/6-1
D
Ethylene (µl/g/hr)
6.0
4.5
3.0
1.5
0
WT
Zmacs2-1
Zmacs6-1
Zmacs2-1/6-1

Figure 2.8. Expression analysis of members of ethylene biosynthetic and signaling gene families in maize roots. RT-PCR analysis of expression of the genes indicated was performed on RNA isolated from the first 1 mm of the apical region of root tips of seedlings grown on moist filter paper (lane 1) or in soil (lane 2) for 14 days. Eukaryotic initiation factor 4A (*eIF4A*) was included as a internal control. For *ZmERS1* and *ZmETR2*, primers were designed to amplify both members of each gene family.

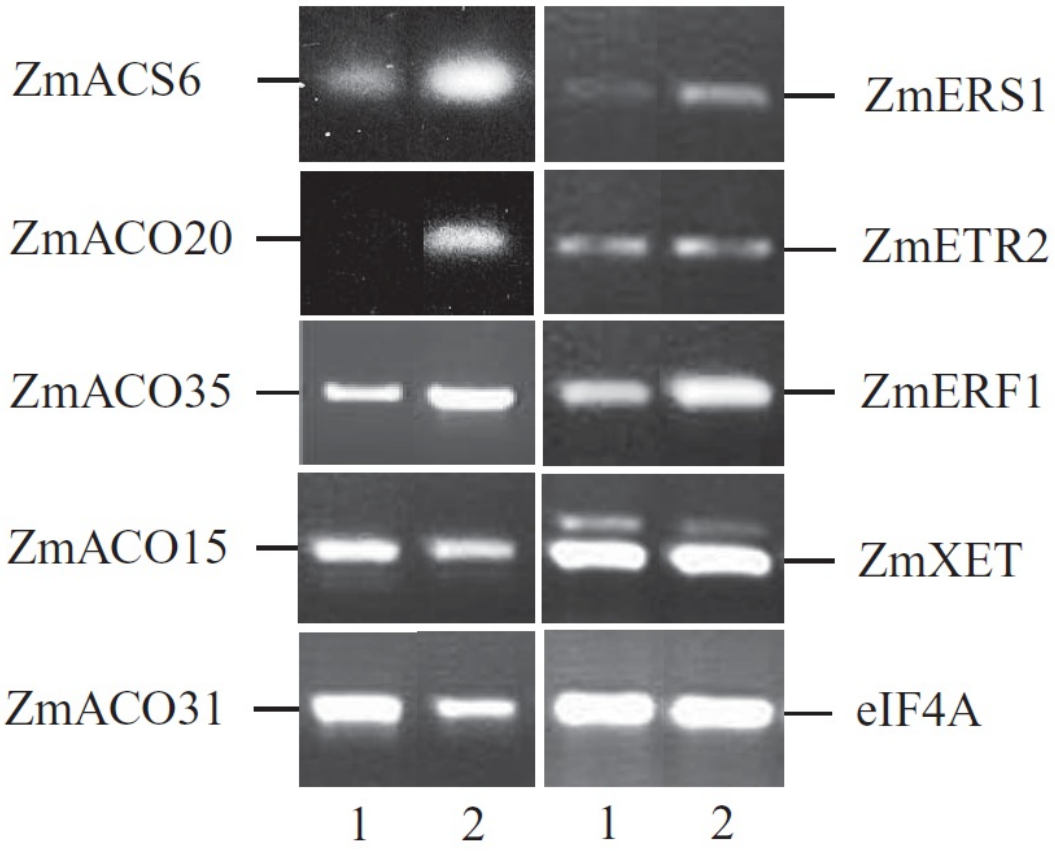

ZmACS6
ZmACO20
ZmACO35
ZmACO15
ZmACO31
ZmERS1
ZmETR2
ZmERF1
ZmXET
eIF4A
1
2
1
2

Table 2.1. Ethylene regulates development of the root cap and metaxylem

	WT	*Zmacs2-1/6-1*	*Zmacs2-1/6-1* + 10 μM ACC
Root cap			
Root cap area (mm^2)[a]	0.123 ± 0.029 (n=16)	0.100 ± 0.006[b] (n=18)	0.110 ± 0.015[c] (n=11)
Maximum cell size (μm^2)[a]	970 ± 212 (n=30)	785 ± 151[b] (n=36)	1099 ± 249[b] (n=31)
Root cap cell number[a]	238 ± 46 (n=30)	312 ± 18[b] (n=26)	218 ± 22[c] (n=31)
Peripheral cell number[a]	10.1 ± 2.4 (n=16)	21.3 ± 5.4[b] (n=15)	11.7 ± 2.8 (n=15)
Vacuolar Component (%)			
Calyptrogen[a]	15.3 ± 12.1 (n=16)	26.0 ± 10.3[b] (n=18)	11.9 ± 4.2 (n=11)
Quiescent center[a]	13.3 ± 7.9 (n=19)	18.1 ± 8.7 (n=11)	11.0 ± 3.0 (n=11)
Root cell dimensions (μm)			
Xylem (longitudinal axis)[a]	13.1 ± 2.07 (n=32)	19.3 ± 3.22[b] (n=33)	14.5 ± 2.30[c] (n=24)
Xylem (transverse axis)[a]	30.1 ± 6.21 (n=32)	32.2 ± 4.83 (n=33)	29.7 ± 3.45 (n=24)
Cortex (longitudinal axis)	10.7 ± 2.33 (n=212)	12.4 ± 1.71[b] (n=70)	10.2 ± 1.78 (n=61)
Cortex (transverse axis)	22.6 ± 3.61 (n=212)	23.6 ± 2.28[c] (n=70)	24.6 ± 1.81[b] (n=61)
Epidermis (longitudinal axis)	8.55 ± 1.79 (n=109)	10.6 ± 2.07[b] (n=60)	9.52 ± 2.30[d] (n=36)
Epidermis (transverse axis)	24.2 ± 2.09 (n=109)	7.5 ± 2.95[b] (n=60)	22.8 ± 0.83[b] (n=36)

[a] As determined from median longitudinal sections

[b] Significantly different from wild type ($P<0.001$)

[c] Significantly different from wild type ($P<0.05$)

[d] Significantly different from wild type ($P<0.01$)

Table 2.2. Ethylene regulates mature root cell size

	WT	*Zmacs2-1/6-1*	*Zmacs2-1/6-1* + 10 μM ACC
Cortical cells			
Longitudinal axis (μm)	104 ± 24.8 (n=101)	177 ± 37.6 [a] (n=124)	120 ± 31.9 [a] (n=142)
Transverse axis (μm)	41.1 ± 4.75 (n=101)	45.3 ± 4.54 [a] (n=124)	41.3 ± 3.24 (n=142)
Epidermal cells			
Longitudinal axis (μm)	102 ± 28.8 (n=37)	142 ± 25.4 [a] (n=30)	113 ± 38.9 (n=50)
Transverse axis (μm)	19.1 ± 1.35 (n=37)	18.0 ± 2.16 [b] (n=30)	18.1 ± 2.86 [b] (n=50)

[a] Significantly different from wild type ($P<0.001$)

[b] Significantly different from wild type ($P<0.05$)

Table 2.3. Ethylene regulates root biomass accumulation of soil-grown maize

	FW (g)	DW (g)	Ethylene (µl/g/hr)
WT (n=12)	75.0 ± 12.3	8.42 ± 1.36	3.30 ± 1.06
Zmacs2-1 (n=12)	74.3 ± 20.4	8.28 ± 2.32 (98.3%)	3.65 ± 0.92
Zmacs6-1 (n=12)	58.0 ± 13.4 [b]	6.77 ± 1.30 [a] (80.4%%)	1.61 ± 0.84
Zmacs2-1/6-1 (n=12)	62.1 ± 16.4 [a]	6.60 ± 1.37 [b] (78.4%%)	1.41 ± 1.15

[a] Significantly different from wild type (P<0.01)

[b] Significantly different from wild type (P<0.005)

CHAPTER 3

Analysis of the Functional Conservation of Ethylene Receptors between Maize and Arabidopsis

ABSTRACT

Ethylene, a plant hormone involved in the regulation of plant growth and development, is perceived by the action of specific receptors which act as negative regulators of the ethylene response. Five different ethylene receptors, i.e., ETR1, ERS1, EIN4, ETR2, and ERS2, have been described in Arabidopsis and dominant negative mutants of each have been isolated or generated that confer ethylene insensitivity to the plant. In contrast, maize contains just two different types of ethylene receptors, ZmERS1 and ZmETR2. In this study, a Cys to Tyr mutation in the transmembrane domain of ZmERS1 and ZmETR2 that is present in the *etr1-1* dominant negative mutant was introduced and each protein was expressed in Arabidopsis. Mutant *Zmers1* and *Zmetr2* receptors conferred a state of ethylene insensitivity and Arabidopsis expressing *Zmers1* or *Zmetr2* were larger and exhibited a delay in leaf senescence characteristic of ethylene insensitive Arabidopsis mutants. *Zmers1* and *Zmetr2* were dominant and functioned equally well in a hemizygous state or homozygous state. Expression of the N-terminal transmembrane domain of *Zmers1* was sufficient to exert dominance over endogenous Arabidopsis ethylene receptors whereas the N-terminal domain from *Zmetr2* failed to do so. Neither *Zmers1* nor *Zmetr2*

functioned in the absence of subfamily 1 ethylene receptors, i.e., ETR1 and ERS1, in Arabidopsis. These results suggest that Cys65 in maize ZmERS1 and ZmETR2 plays the same role that it does for Arabidopsis receptors. Moreover, the results demonstrate that the mutant maize ethylene receptors are functionally dependent on subfamily 1 ethylene receptors in Arabidopsis, indicating substantial functional conservation between maize and Arabidopsis ethylene receptors despite their sequence divergence.

INTRODUCTION

The plant hormone ethylene regulates diverse aspects of plant growth and development, including regulating the rate of germination, seedling growth in the absence of light, sex determination, elongation of the stem or other organs in rice, fruit ripening, organ abscission, leaf and flower senescence, and cell death during cereal endosperm development (1-6). Ethylene also regulates responses to adverse growth conditions, such as hypoxia, mechanical impedance, and pathogen attack (7-15).

Ethylene is produced from methionine in which the latter is converted initially to S-adenosylmethionine (AdoMet) by S-adenosylmethionine synthase, which is then converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). The generation of ethylene results from the oxidation of ACC by ACC oxidase (ACO) in a reaction that also produces CO_2 and HCN (16). Both

ACS and ACO are encoded by multigene families: e.g., the Arabidopsis genome contains nine *ACS* genes which exhibit cell specific and overlapping expression (17-19). The *ACO* gene family may be composed of up to 17 members although not all may function as ACC oxidases (20). The *ACS* and *ACO* gene families are considerably smaller in maize, with just three members comprising the *ZmACS* family and four members comprising the *ZmACO* family (21).

Following its production, ethylene is perceived by binding to endoplasmic reticulum-localized receptors, of which five different types (i.e., ETR1, ERS1, EIN4, ETR2, and ERS2) are present in Arabidopsis (22-29). As negative regulators, the receptors, in conjunction with the CTR1 Raf-like kinase, repress the activity of the downstream components of ethylene signaling in the absence of ethylene (30-32). Binding of ethylene to the N-terminal membrane domain of the receptors relieves the repression of the downstream components of the signaling pathway resulting in the activation of EIN2 and the downstream transcriptional factors including EIN3/EIL and ERF (33-35).

Ethylene receptors share structural similarity with two-component regulators present in bacteria and yeast which are characterized by domains for signal input and output and have His-kinase activity (36-39). In Arabidopsis, ETR1, ETR2, and EIN4 contain a C-terminal receiver domain that follows the His-kinase domain whereas ERS1 and ERS2 do not. ETR1 and ERS1 possess the amino acid sequences and motifs within the His-kinase domain that are

necessary for His-kinase activity and exhibit such activity whereas EIN4, ETR2, and ERS2 lack some or most of these required sequences and instead exhibit Ser-Thr kinase activity, which ERS1 also exhibits (40-45). Because of their demonstrated His-kinase activity and lack of an obvious N-terminal signal peptide, ETR1 and ERS1 have been classified as subfamily I receptors whereas EIN4, ETR2, and ERS2 represent subfamily II receptors (46). Despite the distinction between these two subfamilies based on His-kinase activity, mutants of ETR1 lacking His-kinase activity remain able to rescue the *etr1-7;ers1-2* mutant phenotype in which ETR1 expression is lacking and ERS1 expression is substantially reduced (46). Subfamily I receptors appear to be functionally distinct from subfamily II receptors in that loss of their expression results in a severe constitutive ethylene response (46, 47) and ectopic expression of any subfamily II receptor fails to rescue the *etr1-7;ers1-2* mutant (46). ETR1 has been shown to form covalently linked dimers through a disulfide bond formed between Cys-4 and Cys-6 and the oligomerization of ethylene receptors may play a role in their function (48-50).

Mutations of ethylene receptors resulting in constitutive signaling have been described (31, 40, 41, 51). One such mutant, *etr1-1*, has a Cys to Tyr mutation at residue 65 in the N-terminal transmembrane domain that results in a dominant negative effect and confers a strong ethylene insensitive phenotype (51-53). The N-terminal 349 amino acid residues are sufficient to confer ethylene insensitivity (43). Loss of EIN2 expression also disrupts ethylene signaling and

results in ethylene insensitivity (36). In contrast, loss of CTR1 disrupts the ability of the receptors to repress the activity of the downstream components of the ethylene signaling pathway, resulting in constitutive ethylene signaling.

As with *the ZmACS* and *ZmACO* gene families, the ethylene receptor gene family is smaller in maize with fewer types of ethylene receptors than in Arabidopsis. Maize lacks homologs for ETR1, ERS2, or EIN4 and expresses just two types of ethylene receptors: one with homology to Arabidopsis ERS1 and a second that is the likely homolog of Arabidopsis ETR2 (21). The presence of two genes encoding ZmERS1 (i.e., *ZmERS1a* and *ZmERS1b*) and two genes encoding ZmETR2 (i.e., *ZmETR2a* and *ZmETR2b*) in maize is consistent with the allotetraploid nature of its genome (66, 67). Rice also has two types of ethylene receptors. The subfamily I receptors, OsERS1 and OsERS2, are ERS1-like whereas the subfamily II receptors, OsETR2, OsETR3, and OsETR4, are ETR2-like (68), suggesting that the family structure of ethylene receptors in maize may be a general feature of monocots.

In this report, the conservation of maize ethylene receptor function has been investigated with those in Arabidopsis. A Cys to Tyr mutation was introduced at amino acid 65 in the transmembrane domain of ZmERS1 and ZmETR2 to generate mutant maize receptors that copy the mutation present in the *etr1-1* dominant negative mutant. The effect of the mutation in the maize receptors was determined following their expression in Arabidopsis. Mutant

Zmers1 and *Zmetr2* receptors conferred a state of ethylene insensitivity in Arabidopsis and resulted in many of the phenotypes characteristic of ethylene insensitive Arabidopsis mutants, including increased leaf size and delayed leaf senescence. The mutant maize receptors were dominant and therefore functioned when present in a hemizygous state. Dominance over endogenous Arabidopsis ethylene receptors was observed when just the N-terminal transmembrane domain of mutant *Zmers1* was expressed. Interestingly, expression of the mutant *Zmetr2* N-terminal domain did not confer a state of ethylene insensitivity in Arabidopsis. The mutant maize receptors were dependent on subfamily 1 receptors to function in Arabidopsis as neither *Zmers1* nor *Zmetr2* functioned to confer a state of ethylene insensitivity in the absence of subfamily 1 ethylene receptor expression. These results suggest that Cys65 in maize ZmERS1 and ZmETR2 plays the same role that it does for Arabidopsis receptors and that *Zmers1* or *Zmetr2* are dependent on subfamily 1 ethylene receptors for their function in Arabidopsis, indicating substantial functional conservation between maize and Arabidopsis ethylene receptors.

MATERIALS AND METHODS

Plasmid constructs and mutagenesis

The cDNAs of *ZmETR2* and *ZmERS1* were obtained by RT-PCR from B73 maize RNA and cloned into pGEM-T Easy (Promega, Madison WI, USA). To

generate the *Zmetr2* and *Zmers1* mutants, mutagenesis was performed using the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega, Madison WI, USA). The DNA template was denatured by alkaline treatment, the mutagenic and selection oligonucleotides were annealed, and the mutant strand was synthesized with T4 DNA polymerase and T4 DNA ligase. The DNA was then transformed into BMH 71-18 mutS cells which were grown overnight with the GeneEditor™ Antibiotic Selection Mix. Plasmid DNA was isolated and transformed into JM109 cells. The mutagenic oligonucleotides used were *ZmETR2-40*: 5'-CATCGTGCTCTACGGCCTCACGC-3' and *ZmERS1-25*: 5'-GTTTATAGTTCTCTATGGGGCAAC-3', in which the mutation (i.e., G to A) is underlined. The N-terminal portions of *Zmetr2 (1-386)* and *Zmers1 (1-350)* were obtained by PCR from the full length *Zmetr2* and *Zmers1* constructs. The primer sets used to generate each were *ZmETR2*-F1/*ZmETR2*-R1 and *ZmERS1*-F1/*ZmERS1*-R1, respectively (see below). Both sets of primers were also used for genotyping Arabidopsis containing the *Zmetr2* or *Zmers1* transgenes.

Plant material and transformation

Col-0 Arabidopsis was used throughout this study. After surface-sterilization and cold treatment at 4°C for 4 days in the dark, seeds were planted on 0.25x MS agar plates with or without ACC or $AgNO_3$ at the concentrations indicated. For the triple response assay, seeds were germinated on medium in

the dark for 4 days and the length of the seedling hypocotyl and root measured. For adult plants, seeds were germinated on medium for 1 week and transferred to soil and grown under a 24 h light cycle at 20°C in a plant growth room supplemented with Sylvania Gro-Lite fluorescent bulbs (Sylvania, Danvers MA, USA) at a photon flux density (PFD) of 100 µmol m-2 s-1. Arabidopsis was transformed with each transgene in the binary vector, pBI121, at bolting using Agrobacterium. The primary inflorescence was removed and secondary inflorescences allowed to initiate before infiltration. Inverted plants were dipped into the infiltration medium containing the Aglo1 strain of Agrobacterium containing the transgene. Infiltrated plants were kept on their side for one day and allowed to continue to flower in an upright position in the same growth room. Seeds of infiltrated plants were collected and screened on 0.25x MS plates containing 50 µg/ml kanamycin and 500 µg/ml vancomycin.

Northern analysis

RNA was extracted by quick-freezing plant material in liquid nitrogen, grounding it to a fine powder, and resuspending 100 mg of the material in 1 ml TRIZOL® Reagent (Invitrogen, Carlsbad CA, USA). Following centrifugation, the supernatant was extracted with 200 µl chloroform and centrifuged to separate the phases. The RNA was precipitated from the aqueous phase by isopropyl alcohol, washed with 75% ethanol, resuspended in RNase-free H_2O, and resolved on a

1.2% agarose-formaldehyde gel. PCR-generated Zmetr2 and Zmers1 fragments were radiolabeled with dCTP using Prime-a-Gene labeling system (Promega, Madison WI, USA) and used for hybridization with the membrane overnight at 38°C in 5× SSPE (150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA), 5× Denhardt's solution, 50% formamide, and 1.5% SDS. Blots were washed for 30 min at 45°C in 1× SSPE/0.1% SDS, 30 min at 50°C in 0.5 × SSPE/0.1% SDS, and 30 min at 55°C in 0.2 × SSPE/0.1% SDS. The membrane was then exposed to film at -80°C with an intensifier screen. Each Northern was repeated at least twice. The same membrane was stripped in 50% formamide, 2 × SSPE at 65°C for 30-60 min until no signal could be detected. Where indicated, the membrane was reprobed for *eEF1A*, *PDF1.2*, or *chiB* mRNA using similar conditions.

PCR Analysis

DNA was isolated by quick-freezing plant material in liquid nitrogen, grounding to a fine powder, and resuspending in 400 µl extraction buffer (100 mM Tris-Cl pH 9.0, 20 mM EDTA, 200 mM NaCl, 1 % Sarcosyl, and 1% β-ME). Following centrifugation, the supernatant was extracted with 400 µl phenol: chloroform (1:1) and centrifuged to separate the phases. The DNA was precipitated from the aqueous phase by sodium acetate and isopropyl alcohol, washed with 75% ethanol and resuspended in H_2O. PCR amplification was performed in 20 µl reactions containing 1 x PCR buffer, 0.4 u HotStarTaq DNA

polymerase (Qiagen Inc, Valencia CA, USA), 250 µM dNTPs, 10 µM forward and reverse primers, and 50 ng genomic DNA. Reactions were carried out using the following conditions: 95ºC/5 min (1 cycle); 95ºC/30 sec, 55°C/30 sec, 72ºC/1 min (35 cycles); and a final extension at 72ºC/5 min (1 cycle). To detect the presence of *Zmetr2*, the forward primer, *ZmETR2*-F1, is 5'-ATGGTGGTGGGAACGGCGCCGTGCGGGG-3', and the reverse primer, *ZmETR2*-R1, is 5'-TGCAGTCTGGAAGGAATTCCGAGCTTCC-3'. To detect the presence of *Zmers1*, the forward primer, *ZmERS1*-F1, is 5'-ATGGACGGATGTGATTGCATCGA-3', and the reverse primer, *ZmERS1*-R1, is 5'- AACAGCTAGAAAATCATTGCGAGCACG-3'. To detect the presence of *AtETR1*, the forward primer, *AtETR1*-F1, is 5'-GCGGTTGTTAAGAAATTACCCATCACACT-3', and the reverse primer, *AtETR1*-R1, is 5'-ATCCAAATGTTACCCTCCATCAGATTCAC -3'. To detect the presence of the T-DNA insertion in the *etr1-9* mutant, the forward primer used is *AtETR1*-F1, and the reverse primer, T_DNA-L, is 5'-CATTTTATAATAACGCTGCGGACATCTAC-3'. To detect the presence of wild-type *AtERS1*, the forward primer, *AtERS1*-F1, is 5'-CAGAGAGTTCTGTCACTCCTGGAAATGGT-3', and the reverse primer, *AtERS1*-R1, is 5'-CACAACCGCGCAAGAGACTTTAGCAATAGT-3'. To detect the presence of the T-DNA insertion in the *ers1-3* mutant, the forward primer, *AtERS1*-F2, is 5'-GAACAGGGAATTGTTTCTCAAGAAGAAAGC -3', and the reverse primer is T_DNA-L.

Analysis of etr1-9;ers1-3 double mutants

Seeds of a homozygous *etr1-9*; heterozygous *ers1-3* (i.e., *etr1-9/etr1-9;ers1-3/*+) plant were surface sterilized, cold treated, and germinated on 0.25x MS medium. *etr1-9;ers1-3* double null plants are significantly smaller than plants segregating for the *ers1-3* locus and usually die before flowering. Consequently, these seedlings were removed from the segregating population at this stage. The remaining plants were transferred to soil and grew to flowering under a 24 h light cycle at 20°C. The presence of the *ers1-3* allele was identified by PCR genotyping the adult plants, and *etr1-9/etr1-9;ers1-3/*+ plants were crossed with either the *Zmetr2-9* or *Zmers1-11* lines. Screening for the present of the *ers1-3* allele in F1 progeny from each cross was performed by PCR, and plants that were *T:Zmetr2;etr1-9/+;ers1-3/*+ or *T:Zmers1;etr1-9/+;ers1-3/*+ were allowed to self pollinate. F2 seeds were germinated on 0.25x MS medium and plants exhibiting the small growth phenotype characteristic of *etr1-9;ers1-3* plants were genotyped by PCR analysis to determine the presence of each maize transgene as well as the presence of the *ETR1*, *etr1-9*, *ERS1*, and *ers1-3* loci.

RESULTS

Dominant negative Zmetr2 and Zmers1 mutants confer ethylene insensitivity in Arabidopsis

ZmERS1a and ZmERS1b, encoded by *ZmERS1a* and *ZmERS1b* (originally designated *ZmERS1-14* and *ZmERS1-25*, respectively) are 96% identical at the amino acid level (21). ZmETR2a and ZmETR2b, encoded by *ZmETR2a* and *ZmETR2b* (originally designated *ZmETR2-9* and *ZmETR2-40*, respectively) are 92% identical at the amino acid level of the mature protein (21). The ZmERS1 receptors are similar to Arabidopsis ERS1 in that they contain an N-terminal domain composed of three transmembrane spanning regions, followed by a GAF domain and a His-kinase domain that possesses the amino acid sequences and motifs required for His-kinase activity, but lack a C-terminal receiver domain (Fig. 3.1.A). ZmETR2 receptors are similar to Arabidopsis ETR2 in that they contain an N-terminal domain composed of four transmembrane spanning regions, followed by a GAF domain, a His-kinase domain that lacks several of the amino acid sequences and motifs required for His-kinase activity, and a C-terminal receiver domain (Fig. 3.1.A).

In order to determine the extent to which the maize ethylene receptors are functionally conserved with those of Arabidopsis, the sequences of *ZmETR2b* and *ZmERS1b* were used to amplify the open reading frame of each gene from the inbred B73. The sequence of each was then mutated at the codon

corresponding to Cys65 in Arabidopsis ETR1 to change the specified amino acid from Cys to Tyr, as in the *etr1-1* mutant receptor, resulting in the generation of the *Zmetr2b* and *Zmers1b* mutants. The *Zmetr2b* and *Zmers1b* coding regions were placed under the control of the 35S promoter in pBI121 for *Agrobacterium*-mediated transformation of Arabidopsis, from which several independent transformants homozygous for each transgene were isolated. The presence of the *Zmetr2b* or *Zmers1b* transgene in candidate transformant lines was confirmed by PCR (Fig. 3.1.B).

To investigate the induction of *Zmetr2b* or *Zmers1b* expression and ethylene insensitivity, three independent transformant lines homozygous for either *Zmetr2b* (i.e., lines L4, L5, L9) or *Zmers1b* (i.e., lines L11, L12, L15) were germinated and RNA was extracted from 10 day old seedlings for Northern analysis. Expression of *Zmetr2b* was readily observed in each of the lines (lanes 2-4, top panel, Fig. 3.1.C). Expression of *Zmers1b* in lines L11 and L15 appeared lower than Zmetr2b expression and was observed only upon longer exposure (lanes 5-7, middle panel, Fig. 3.1.C), despite similar levels of total RNA loaded as determined by the transcript abundance of translation elongation factor 1A (*eEF1A*) mRNA, which was used as the internal control (bottom panel, Fig. 3.1.C). Little to no *Zmers1b* expression was observed in line L12 (lane 7, middle panel, Fig. 3.1.C). The *Zmetr2b* or *Zmers1b* probes used for the Northern analysis did not detect the expression of Arabidopsis ethylene receptors (lanes 1, 8-9, middle panel, Fig. 3.1.C).

To determine whether expression of *Zmetr2b* or *Zmers1b* could repress ethylene responses in Arabidopsis, the triple response of transgenic seedlings was examined when grown on medium containing ACC, the precursor to ethylene. The triple response in Arabidopsis is an ethylene-mediated response of dark-grown seedlings characterized by the radial expansion of the hypocotyl, inhibition of root and hypocotyl elongation, and the presence of an exaggerated apical hook (54). Wild-type (WT) seedlings grown in the dark on 20 μM ACC exhibited these characteristics (top panel, Fig. 3.1.D). Hypocotyl growth in seedlings expressing *Zmetr2b*, however, was substantially greater than WT seedlings and was similar to the growth of the ethylene insensitive mutant, *ein2-5* (top panel, Fig. 3.1.D and Table 3.1). Root growth in lines expressing *Zmetr2b* was greater than WT seedlings but slightly reduced relative to *ein2-5* roots (Table 3.1). No difference in the growth of WT seedlings, the *ein2-5* mutant, or lines expressing *Zmetr2b* was observed during growth on 5 μM Ag^{2+}, which inhibits ethylene perception by likely replacing the copper cofactor in receptors and uncoupling ethylene binding from signal output (55). Similar results were obtained for lines expressing *Zmers1b* in that a lack of a triple response was observed for lines expressing *Zmers1b* (i.e., L11 and L15) when grown in the dark on 20 μM ACC and that root growth was slightly reduced relative to that observed for the *ein2-5* mutant (top panel, Fig. 3.1.D and Table 3.1). Ethylene insensitivity in the *Zmers1b*-expressing lines was observed despite the apparent lower level of *Zmers1b* expression relative to that of *Zmetr2b* expression in

Zmetr2b-expressing lines, suggesting that the amount of *Zmers1b* receptor produced was sufficient to confer ethylene insensitivity. In *Zmers1b* L12, however, only a slight reduction in ethylene sensitivity was observed (top panel, Fig. 3.1.D and Table 3.1), which correlated with the lower level of *Zmers1b* expression than in the other lines, i.e., L11 and L15.

In light-grown seedlings, exposure to elevated levels of ethylene inhibits cotyledon expansion, represses root growth, and delays the emergence of true leaves (56). Light-grown WT seedlings exhibited these characteristics in the presence of 20 µM ACC whereas growth was normal in the absence of ACC (Fig. 2.2.A). In *Zmetr2b*-expressing seedlings, cotyledon expansion and the emergence of the first true leaves were not as inhibited by growth on 20 µM ACC as in WT seedlings and was similar to that observed *in ein2-5* and *etr1-1* seedlings (Fig. 2.2.A). Root growth in *Zmetr2b*-expressing seedlings grown in the presence of 20 µM ACC was greater than in WT seedlings but less than in *ein2-5* and *etr1-1* seedlings. qPCR analysis of light-grown, *Zmetr2b*-expressing seedlings (line L9) demonstrated that *Zmetr2b* expression in roots was 1.7% of the level in leaves (Fig. 2.2.B), correlating with the observed lower level of ethylene insensitivity in its roots. Cotyledon expansion and the emergence of the first true leaves in *Zmers1b*-expressing seedlings were also greater than that of WT seedlings in the presence of 20 µM ACC (Fig. 2.2.A). Root growth was also greater than in WT seedlings but less than *in ein2-5* seedlings and was greater than in *Zmetr2b*-expressing seedlings (Fig. 2.2.A). qPCR analysis of light-grown,

Zmers1b-expressing seedlings (line L11) demonstrated that *Zmers1b* expression in roots was 23.2% of the level in leaves (Fig. 2.2.B), consistent with the greater level of root growth observed *in Zmers1b* seedlings relative to *Zmetr2b* seedlings (Fig. 2.2.A). In line L12, seedling growth was similar to WT seedlings in the presence of 20 μM ACC, supporting the conclusion that there was, at best, a slight reduction in ethylene sensitivity in this line, correlating with its low level of *Zmers1b* expression.

Growth of the *Zmetr2b* and *Zmers1b*-expressing lines was followed throughout their life cycle. A delay in flowering and an increase in rosette size have been reported for *ein2-5* (52). An increase in leaf number and a delay in flowering were observed for *Zmetr2b* and *Zmers1b*-expressing lines, especially for *T:Zmetr2b* line L9 (Table 2.2). No significant change in chlorophyll content or in the chlorophyll a/b ratio was observed for the *Zmetr2b* and *Zmers1b*-expressing lines or the *ein2-5* mutant for 3 week-old plants (Table 2.2) but an increase in leaf size (Fig. 3.3.A) and a delay in leaf senescence (Fig. 3.3.B) was observed for *Zmetr2b* and *Zmers1b*-expressing lines as was observed for the *ein2-5* and *etr1-1* mutants. The increase in leaf size and delay in leaf senescence correlated with the degree of ethylene insensitivity exhibited by each line. For example, *T:Zmetr2b* line L9 and *T:Zmers1b* line L11, both of which were highly ethylene insensitive, had substantially larger adult leaves (Fig. 3.3.C) and a pronounced delay in leaf senescence whereas leaf size and rate of leaf

senescence in *T:Zmers1b* line L12, which had exhibited only a slight reduction in ethylene sensitivity, was similar to WT plants (Fig. 3.3).

To determine whether expression of the *Zmetr2b* and *Zmers1b* transgenes would exert dominance when present in a hemizygous state, *T:Zmetr2b* line L9 and *T:Zmers1b* line L11 were crossed with WT plants to generate seed in which each transgene was present in a hemizygous state and their level of ethylene insensitivity in the triple response assay compared to lines homozygous for *Zmetr2b* or *Zmers1b*. When grown in the presence of 20 μM ACC, hemizygous *Zmetr2b* or *Zmers1b* seedlings exhibited a similar degree of ethylene insensitivity as seedlings homozygous for *Zmetr2b* or *Zmers1b* as determined by growth of the hypocotyl and root and the lack of an apical hook (Fig. 3.4 and Table 3.3). A slight reduction in root growth in hemizygous *Zmetr2b* seedlings relative to roots of homozygous *Zmetr2b* seedlings was observed (Table 3.3). These results suggest that expression of *Zmetr2b* and *Zmers1b* can exert dominance when present in a hemizygous state.

Segregation of the ethylene insensitivity phenotype in F2 progeny from a hemizygous *Zmetr2b* or a hemizygous *Zmers1b* parent was also performed to determine genetically the copy number of each transgene. Of 156 *Zmetr2b* progeny analyzed, 123 were ethylene insensitive and 33 were ethylene sensitive as determined by grown in the light in the presence of 20 μM ACC. Of 149 *Zmers1b* progeny analyzed, 114 were ethylene insensitive and 35 were ethylene

sensitive. This represents a segregation 3.7:1 ratio for the *Zmetr2b* transgene and a 3.26 ratio for the *Zmers1b* transgene which, for a dominant phenotype, suggests that each transgene is present as a single copy.

Expression of Zmetr2b or Zmers1b Confers a High Level of Insensitivity to Ethylene

To determine the degree to which expression of *Zmetr2b* and *Zmers1b* confers a state of ethylene insensitivity, the growth *of T:Zmetr2b* line L9 and *T:Zmers1b* line L11 seedlings was compared to WT seedlings in the triple response assay on medium containing different levels of ACC. Hypocotyl growth was greatest for WT seedlings grown in the presence of Ag^{2+} (Fig. 3.5), which was confirmed by quantitative measurements (Fig. 3.6). In the absence of Ag^{2+}, hypocotyl growth of WT seedlings was reduced relative to growth on Ag^{2+}. Significant inhibition of hypocotyl and root growth and a prominent apical hook in WT seedlings was observed at 1 µM ACC (Fig. 3.5) and full inhibition was achieved by approximately 2.5 µM ACC (Fig. 3.6).

Hypocotyl growth of *T:Zmetr2b* line L9 and *T:Zmers1b* line L11 seedlings was slightly less than that in WT seedlings in the presence of Ag^{2+} but greater than that in WT seedlings in the absence of Ag^{2+} (Fig. 3.6). The growth of the hypocotyl in *T:Zmetr2b* line L9 and *T:Zmers1b* line L11 seedlings was largely unaffected by increasing ACC concentrations up to 5 µM and no apical hook was evident (Fig. 3.5). A slight reduction in hypocotyl growth was observed in

T:Zmers1b line L11 seedlings at higher ACC concentrations (Fig. 3.6) with a few seedlings exhibiting an apical hook (Fig. 3.5). *T:Zmetr2b* line L9 roots were slightly shorter than WT roots when grown in the absence of ACC but their growth was not inhibited to same extent as in WT seedlings by increasing concentrations of ACC (Fig. 3.6). In contrast, *T:Zmers1b* line L11 root growth was inhibited to a similar extent as WT roots in the presence of increasing concentrations of ACC (Fig. 3.6). These results demonstrate that the state of ethylene insensitivity in the hypocotyl conferred by *Zmetr2b* and *Zmers1b* expression is maintained over a wide range of ACC concentrations with a reduction in sensitivity to ethylene in roots.

Expression of *Zmetr2b* and *Zmers1b* in Arabidopsis conferred insensitivity to ethylene as measured by the lack of ethylene-mediated hypocotyl growth inhibition. To examine whether the dominance of *Zmetr2b* and *Zmers1b* mutant expression also inhibited ethylene responses at the molecular level, the expression of genes known to be ethylene inducible was examined. Light-grown *T:Zmetr2b* line L9 and *T:Zmers1b* line L11 plants were treated with 100 ppm ethylene for 24 hours while additional plants were maintained in air for the same period to serve as an air-treated control. Total RNA was extracted from both the ethylene and air-treated plants for Northern analysis. Expression of *Zmetr2b* and *Zmers1b* mRNA was somewhat higher in ethylene-treated plants relative to air-treated plants when normalized to *eEF1A* mRNA, which was used as the RNA loading control (Fig. 3.7). As observed in Fig. 3.1, the *Zmetr2b* or *Zmers1b*

probes did not cross react with Arabidopsis ethylene receptor mRNA as demonstrated in WT plants (Fig. 3.7). Expression from *chiB* and *PDF1.2*, two ethylene-inducible genes in adult Arabidopsis leaves, was absent in air-treated WT plants but was induced in ethylene-treated plants (Fig. 3.7). No expression of either gene was observed in *Zmetr2b* L9 or *Zmers1b* L11 plants in the presence or absence of ethylene or in *ein2-5* or *etr1-1* plants as would be expected for these ethylene insensitive mutants (Fig. 3.7). These results suggest that *Zmetr2b* or *Zmers1b* expression represses the induction of ethylene-regulated genes.

The N-terminal Domain of Zmers1b but not Zmetr2b is Sufficient to Confer Ethylene Insensitivity

Expression of the N-terminal 349 amino acids of *etr1-1*, i.e., *etr1-1(1-349)* can confer ethylene insensitivity in Arabidopsis (43). To determine whether the N-terminal domain of maize ethylene receptors containing the C65Y mutation is sufficient to exert dominance over the Arabidopsis receptors, the portion of the *Zmetr2b* and *Zmers1b* coding region corresponding to *etr1-1(1-349)* was placed under the control of the 35S promoter in pBI121 for *Agrobacterium*-mediated transformation of Arabidopsis, from which several independent transformants homozygous for each transgene were isolated. The presence of the *Zmetr2b(1-386)* or *Zmers1b(1-350)* in the candidate transformants was confirmed by PCR (data not shown). To determine whether expression of *Zmetr2b(1-386)* or

Zmers1b(1-350) was able to confer ethylene insensitivity, seeds from three independent transformed lines for each transgene were germinated in the presence of 20 μM ACC to examine the degree of their triple response. Expression of full-length *Zmetr2b* resulted in a level of ethylene insensitivity similar to that in *ein2-5* or *etr1-1* seedlings (Fig. 3.8.A) as observed previously. Expression of *Zmetr2b(1-386)* was unable to confer ethylene insensitivity in the three independent transformed lines examined. This failure was not a result of a lack of *Zmetr2b(1-386)* expression as its expression was easily detected in at least one of the three lines (Fig. 3.8.C). In contrast, expression of *Zmers1b(1-350)* was sufficient to exert dominance over the Arabidopsis receptors in all three of the transformed lines tested (Fig. 3.8.B) and in which *Zmers1b(1-350)* expression was easily detected (Fig. 3.8.C). These results demonstrate that the N-terminal domain of the *Zmers1* receptor but not the *Zmetr2* receptor is sufficient to confer ethylene insensitivity in Arabidopsis.

Zmetr2b and Zmers1b Function is Dependent on Subfamily 1 Expression in Arabidopsis

The function of *etr1-1* as a dominant negative regulator of ethylene signaling is dependent on the expression of subfamily 1 receptors, which, in Arabidopsis, includes ETR1 and ERS1 (57). To examine whether *Zmetr2b* or *Zmers1b* exert their dominance through subfamily 1 receptors, the ability of each

mutant maize receptor to confer ethylene insensitivity was tested in *etr1-9;ers1-3*, a double knockout mutant that does not express ETR1 or ERS1 (58). Loss of ETR1 and ERS1 expression results in growth phenotypes more severe than those observed for *ctr1*, which itself is characterized by constitutive ethylene signaling (30). The *etr1-9;ers1-3* double mutant plant is extremely small and typically dies before flowering (58), precluding crosses with this mutant. As a consequence, the two mutations are typically maintained in plants containing the *etr1-9* mutation in a homozygous state and the *ers1-3* mutation in a heterozygous state, which are viable and fertile (58). The *Zmetr2b* or *Zmers1b* transgene was introduced into the *etr1-9;ers1-3*/+ mutant through crosses with *T:Zmetr2b* line L9 *or T:Zmers1b* line L11, generating F1 progeny that were hemizygous for either *Zmetr2b* or *Zmers1b*, heterozygous for *etr1-9*, and either heterozygous for *ers1-3* or homozygous for *ERS1*. F1 progeny identified as *ers1-3/*+ by PCR genotyping were selfed and F2 seed germinated in the light. Those F2 progeny exhibiting the extremely small growth phenotype typical of *etr1-9;ers1-3* plants were genotyped by PCR to verify the presence of the *etr1-9* and *ers1-3* mutations as well as the absence of the corresponding wild-type loci. The same plants were also genotyped to determine the presence of either the *Zmetr2b* or *Zmers1b* transgene. If either *Zmetr2b* or *Zmers1b* functioned to confer a state of ethylene insensitivity to *etr1-9;ers1-3* plants, then neither the *Zmetr2b* nor the *Zmers1b* transgene would be detected in *etr1-9;ers1-3* F2 progeny exhibiting the extremely small growth phenotype. If, however, *Zmetr2b*

or *Zmers1b* failed to function in *etr1-9;ers1-3* plants (i.e., in the absence of ETR1 and ERS1 expression), the *Zmetr2b* or *Zmers1b* transgene would be expected to segregate in *etr1-9;ers1-3* F2 progeny exhibiting the extremely small growth phenotype. Analysis of small F2 progeny from crosses between *T:Zmetr2b* line L9 and *etr1-9;ers1-3*/+ plants revealed the presence of the *Zmetr2b* transgene (Table 3.5). F2 progeny containing *Zmetr2b* included plants that were *etr1-9;ers1-3* (Table 3.5) and such plants were substantially smaller than plants exhibiting wild type growth (Fig. 3.9). Similar results were obtained for the *Zmers1b* transgene in that *Zmers1b* was present in several small F2 progeny from crosses between *T:Zmers1b* line L11 and *etr1-9;ers1-3*/+ plants and that F2 progeny containing *the Zmers1b* transgene included plants that were *etr1-9;ers1-3* (Table 3.5). Such plants were substantially smaller than plants exhibiting wild type growth (Fig. 3.9). These results demonstrate that *Zmetr2b* and *Zmers1b* fail to rescue the small growth phenotype of the *etr1-9;ers1-3* double mutant, indicating that the function of *Zmetr2b* and *Zmers1b* receptors is dependent on the expression of subfamily 1 members.

DISCUSSION

Based on sequence conservation and domain structure, maize expresses only two types of ethylene receptors, i.e., ZmERS1 and ZmETR2, in contrast to the five types of receptors expressed in Arabidopsis. In this study, it is shown that, despite the difference in sequence and types of receptors in the two species, maize receptor function is conserved in Arabidopsis. Introducing the same C65Y mutation into ZmERS1b and ZmETR2b that is present in the *etr1-1* dominant negative mutant resulted in dominant negative mutant receptors that conferred ethylene insensitivity in Arabidopsis. Plants expressing *Zmers1b* or *Zmetr2b* exhibited many of the phenotypes associated with ethylene insensitive Arabidopsis mutants, including a lack of a triple response when dark-grown seedlings were germinated in the presence of ACC, a larger leaf size and a delay in leaf senescence in light-grown plants, and repression of ethylene-inducible gene expression. *Zmers1b* and *Zmetr2b* conferred a state of ethylene insensitivity in Arabidopsis seedlings when present in either a hemizygous or a homozygous state. Rice also expresses only ERS1-like and ETR2-like receptors (59), suggesting that the perception of ethylene in monocots may be limited to these two receptor types. Thus, the observations made with the maize ethylene receptors in this study may have broad applicability to monocots in general.

Expression of *Zmers1b* and *Zmetr2b* in Arabidopsis resulted in a level of ethylene insensitivity in the hypocotyl of dark-grown seedlings or in leaves of

light-grown seedlings comparable to that in *ein2-5* or *etr1-1* mutants, but in roots, they conferred only partial insensitivity, correlating with their lower expression in roots relative to leaves. The native maize promoter was not used to express *Zmers1b* and *Zmetr2b* as there was no assurance that either monocot promoter would function appropriately in Arabidopsis to provide a “native” level of expression. Moreover, the use of an Arabidopsis ethylene receptor promoter to express the maize receptors would not assure a “native” level of expression as the stability and translational efficiency of maize receptor mRNAs and their protein stability will contribute to their steady state level of expression. Using the 35S promoter to express wild-type ZmERS1b and ZmETR2b receptors did not result in an observable phenotype in Arabidopsis (data not shown), suggesting that the level of expression of *Zmers1b* and *Zmetr2b* in Arabidopsis is not due to unusual expression patterns or levels. These observations are consistent with the conclusion that maize *Zmers1b* or *Zmetr2b* receptors function in Arabidopsis and that the C65Y mutation has a similar effect in both maize receptors.

How such C65Y mutants, best studied in *etr1-1*, exert dominance over endogenous receptors is not fully understood. The C65Y mutation in *etr1-1* perturbs binding of the Cu cofactor at this site, preventing binding of ethylene (55, 60). This is thought to maintain the mutant receptor in a state that constitutively represses activation of the downstream components of the signaling pathway, e.g., EIN2 and EIN3 (43, 57). Thus, binding of ethylene to wild type receptors fails to activate an ethylene response in the presence of *etr1-1* that constitutively

represses ethylene responses. This suggests that the dominance of *etr1-1* resides in its ability to constitutively repress ethylene responses whether or not ethylene is present by maintaining output signaling to the downstream components of the signaling pathway.

Expression of *etr1-1(1-349)*, which lacks the His-kinase and receiver domains, is sufficient to repress ethylene responses (43), suggesting that this region is responsible for output signaling itself or that it exerts its dominance through interaction with wild-type receptors. The observation that *etr1-1(1-349)* functions to repress ethylene responses in an *etr1-7;ers1-2* mutant, in which a low level of ERS1 expression remains, but not in an *etr1-7;ers1-3* mutant, in which no ERS1 expression is detectable, supports the notion that *etr1-1(1-349)* requires expression of subfamily I members (57). Expression of *Zmers1b(1-350)* was sufficient to cause ethylene insensitivity in Arabidopsis, demonstrating that the putative His-kinase domain is not required for *Zmers1b* function. Although the ability of the N-terminal region of an Arabidopsis *ers1*(C65Y) mutant to function in ethylene signaling has not been reported, given the similarity between ETR1 and ERS1 and that the major difference between the two, i.e., the presence of the receiver domain in ETR1 and its absence in ERS1, is lacking in *etr1-1(1-349)*, it is possible that an N-terminal *ers1*(C65Y) mutant may be capable of repressing ethylene signaling. The observation that *Zmers1b(1-350)* was sufficient to cause ethylene insensitivity in Arabidopsis indicates that the N-terminal region of the ERS1 class of receptors is capable of output signaling, either directly or through

interactions with endogenous receptors, when the C65Y mutation is present. Interestingly, expression of *Zmetr2b(1-386)* was unable to cause ethylene insensitivity, suggesting that the truncated peptide was not capable of ethylene output signaling, either directly or indirectly, despite the fact that full-length *Zmetr2b* provided strong ethylene signaling. The failure of *Zmetr2b(1-386)* to repress ethylene responses was not due to a lack of expression as it was readily detected by Northern analysis. If the function of the N-terminal region of receptor mutants, such as *etr1-1(1-349)* or *Zmers1b(1-350)*, is mediated through interactions with endogenous receptors, the extent of their conservation with endogenous receptors may determine their interaction and therefore their effectiveness. ZmERS1 is approximately 73% identical with Arabidopsis ERS1 but ZmETR2 is only 45% identical with Arabidopsis ETR2 (21). The difference in conservation with their respective Arabidopsis subfamily receptors may account for the difference in the ability of the *Zmers1b(1-350)* and *Zmetr2b(1-386)* peptides to exert dominance. It is possible, however, that the inability of *Zmetr2b(1-386)* to repress ethylene responses is a result of the instability of the peptide or its inability to fold correctly. It is also possible the C-proximal sequence missing in *Zmetr2b(1-386)* is required for the dominant function exhibited by the full-length *Zmetr2* mutant receptor.

Zmers1b and *Zmetr2b* failed to confer ethylene insensitivity in the *etr1-9;ers1-3* mutant, demonstrating that the function of both mutant receptors requires expression of subfamily I members in Arabidopsis. These observations

suggest that *Zmers1b(1-350)* may function through an interaction with subfamily I members to exert its dominance as proposed for *etr1-1(1-349)* (57), indicating that the N-terminal region of *Zmers1* is sufficiently conserved with Arabidopsis subfamily I receptors to permit the functional interaction needed to repress ethylene responses. Such a functional interaction for *etr1-1(1-349)* may involve maintaining subfamily I receptors in a signaling state or that the interaction with subfamily I receptors permits signaling from the *etr1-1(1-349)* truncated protein itself (57).

ETR1 in Arabidopsis can form covalently linked dimers through a disulfide bond formed between Cys-4 and Cys-6 which may be involved in ethylene signaling (43, 48, 50, 61). *etr1-1(1-349)* covalently dimerizes with ETR1 (43), demonstrating that the C65Y mutation does not disrupt its interaction with the wild-type receptor. Although mutation of Cys-4 and Cys-6 *in etr1-1(1-349)* did not abolish N-terminal signaling, the dominant signaling from the truncated receptor was reduced (57), raising the possibility that the interaction mediated through the disulfide bonds may contribute to signaling. The maize ZmERS1 and ZmETR2 receptors share structural similarity with the Arabidopsis subfamily I and II receptors, respectively. The Cys-4 and Cys-6 present in ETR1 and ERS1 are conserved in ZmERS1b and in ZmETR2b (21). In ZmETR2b, the cysteines are C-proximal to a putative signal peptide as they are in Arabidopsis subfamily II receptors. Whether any interaction between ZmERS1b and subfamily I receptors requires the formation of disulfide bonds between maize and Arabidopsis

receptors remains to be determined. The observation that mutation of Cys-4 and Cys-6 in *etr1-1(1-349)* did not abolish N-terminal signaling (57), however, suggests that the interaction between receptors may also be facilitated by non-covalent interactions.

An observed interaction between ETR1 and ERS2 in Arabidopsis was largely disrupted by SDS treatment, indicating their association is maintained by higher order interactions although 20% of the heterodimers was resistant to the treatment suggesting that the formation of disulfide bonds may contribute to receptor association (50, 62). These findings suggest that receptor interactions are largely maintained through higher order interactions that may include non-covalent interactions between GAF domains, which in other two-component regulators, can dimerize (63-65). The GAF domain may function similarly in ethylene receptors as the GAF domain is sufficient to mediate the interaction between Arabidopsis ETR1 and ETR2 (62). These results also demonstrate cross interactions between ethylene receptor subfamilies. The GAF domain is present in the *Zmers1b(1-350)* and *Zmetr2b(1-386)* peptides (Fig. 3.1.A). The ZmERS1b GAF domain exhibits a high level of conservation with Arabidopsis subfamily I receptor GAF domains whereas the conservation between the GAF domain of ZmETR2b and Arabidopsis subfamily II receptor GAF domains is considerably lower (21). An investigation into the extent to which the GAF domain determines interactions between ethylene receptors may provide greater insight into receptor function, particularly for dominant receptor mutations.

In conclusion, the analysis of maize ethylene receptors in Arabidopsis has revealed considerable functional conservation in the role that Cys65 plays in ethylene signaling, the ability of the full-length *Zmers1b* and *Zmetr2b* receptors or *Zmers1b(1-350)* to repress ethylene responses, and the dependence of *Zmers1b* and *Zmetr2b* on subfamily 1 ethylene receptors for their function.

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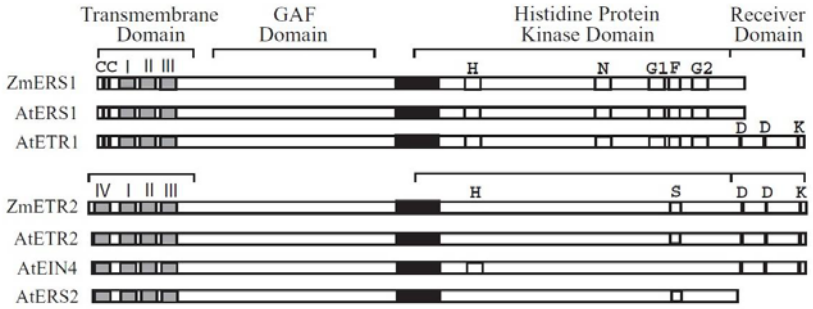
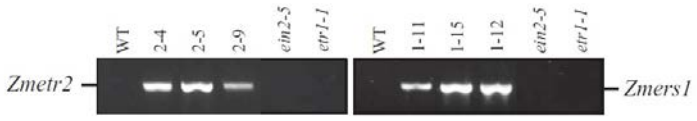
Figure 3.1. *Zmetr2* and *Zmers1* function as dominant negative mutants in Arabidopsis. (A) Comparison of the *Zmers1* receptor with Arabidopsis subfamily I receptors, i.e., AtETR1 and AtERS1, and comparison of the *Zmetr2* receptor with Arabidopsis subfamily II receptors, i.e., AtETR2, AtEIN4, and AtERS2. The N-terminal, hydrophobic, transmembrane domains are indicated by gray boxes. Cys-4 and Cys-6, are indicated by the Cs at the left end of the proteins. The five consensus motifs (H, N, G1, F, and G2) within the histidine protein kinase domain (31) are indicated, and the aspartate and lysine residues conserved in the receiver domain of ETR1 are indicated. The serine-rich domain (S) is also indicated. The proposed coiled-coiled region is indicated by the large black box. (B) PCR amplification of the *Zmetr2* and *Zmers1* transgenes from three lines containing *Zmetr2* (i.e., 2-4, 2-5, 2-9) and three lines containing *Zmers1* (i.e., 1-11, 1-15, 1-12) confirming the presence of the transgene in the transformants. Wild-type (WT), *ein2-5*, and *etr1-1* plants were included as negative controls. (C) Northern analysis of seedlings of the same *Zmetr2* or *Zmers1* lines germinated in the dark for 10 days. The level of *Zmetr2* and *Zmers1* expression was measured using a mixture of *Zmetr2* and *Zmers1* probes after a 24 hr (top panel) or 2 week (middle panel) exposure of the membrane to film. Expression of the translation elongation factor 1A (*eEF1A*) mRNA was determined as a RNA loading control from the same membrane after it had been stripped (bottom panel). (D) Seeds from the same lines were germinated in the dark for 5 days on media containing either 20 μM ACC or 5 μM $AgNO_3$ to assay for their triple response. Two representative seedlings are shown for each line. Quantitative measurements for hypocotyl and root lengths with standard deviations are shown in Table 3.1.

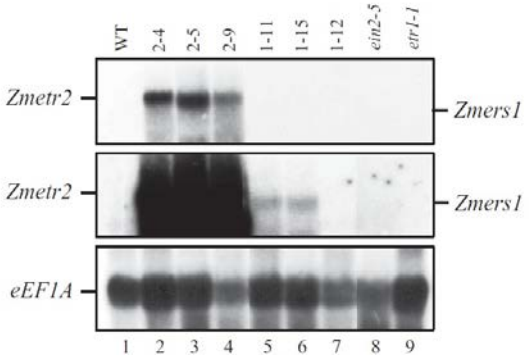
A


Transmembrane Domain
GAF Domain
Histidine Protein Kinase Domain
Receiver Domain
CC I II III
H
N
G1F G2
ZmERS1
AtERS1
AtETR1
D D K
IV I II III
H
S
D D K
ZmETR2
AtETR2
AtEIN4
AtERS2

B


WT
2-4
2-5
2-9
ein2-5
etr1-1
WT
1-11
1-15
1-12
ein2-5
etr1-1
Zmetr2
Zmers1

C


WT
2-4
2-5
2-9
1-11
1-15
1-12
ein2-5
etr1-1
Zmetr2
Zmers1
Zmetr2
Zmers1
eEF1A
1 2 3 4 5 6 7 8 9

D

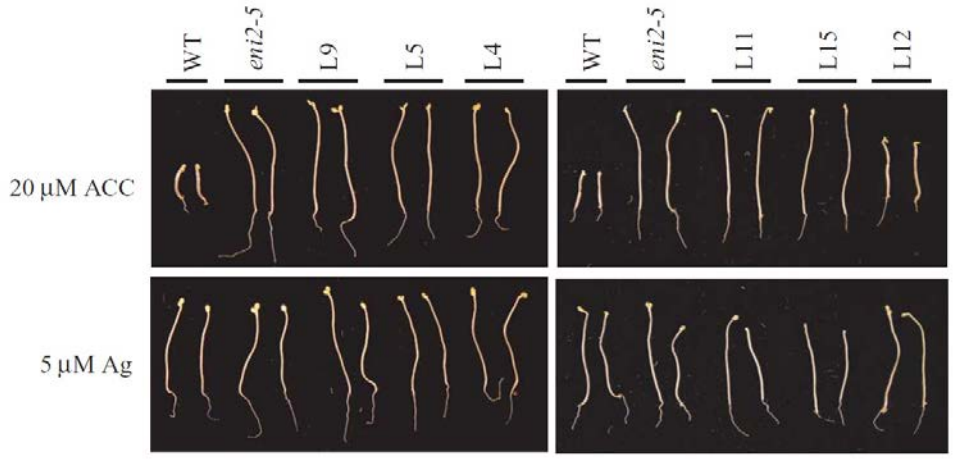
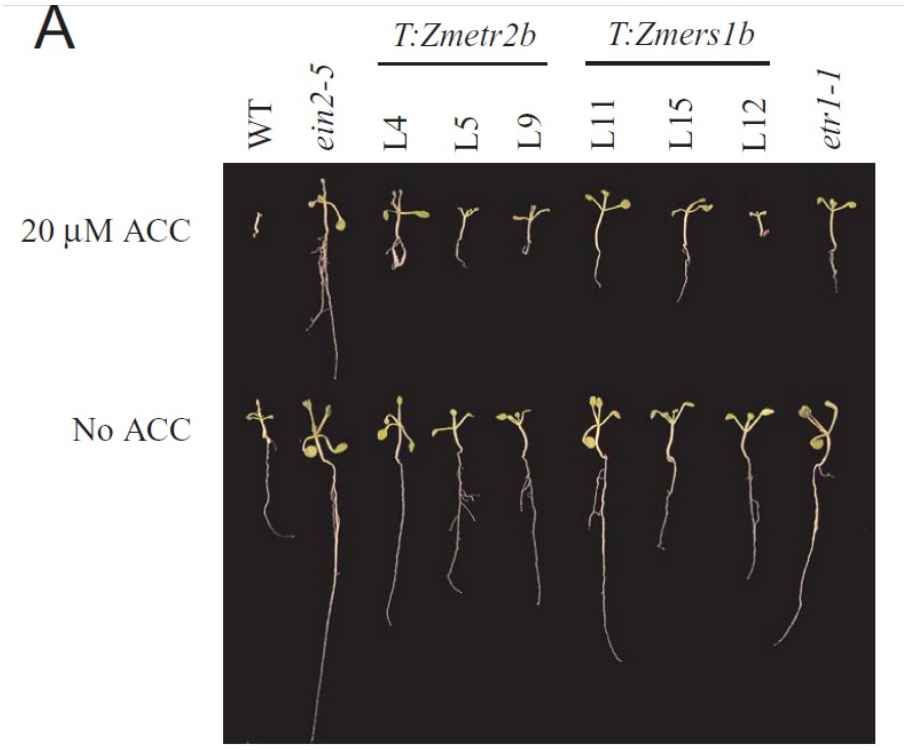

WT
eni2-5
L9
L5
L4
WT
eni2-5
L11
L15
L12
20 µM ACC
5 µM Ag

Figure 3.2. Expression of *Zmetr2b* and *Zmers1b* confers ethylene insensitivity in light-grown Arabidopsis seedlings. In (A), the same lines used in Figure 3.1 were germinated in the light for 10 days on media in the presence or absence of 20 μM ACC. One representative seedling is shown for each line. In (B), qPCR analysis of leaf and root tissue of light-grown seedlings of lines *T:Zmetr2b* L9 and *T:Zmers1b* L11. The level of expression of each transgene in leaves was set to a value of one and transgene expression in roots is shown relative to that in leaves.


A
T:Zmetr2b
T:Zmers1b
WT
ein2-5
L4
L5
L9
L11
L15
L12
etr1-1
20 µM ACC
No ACC

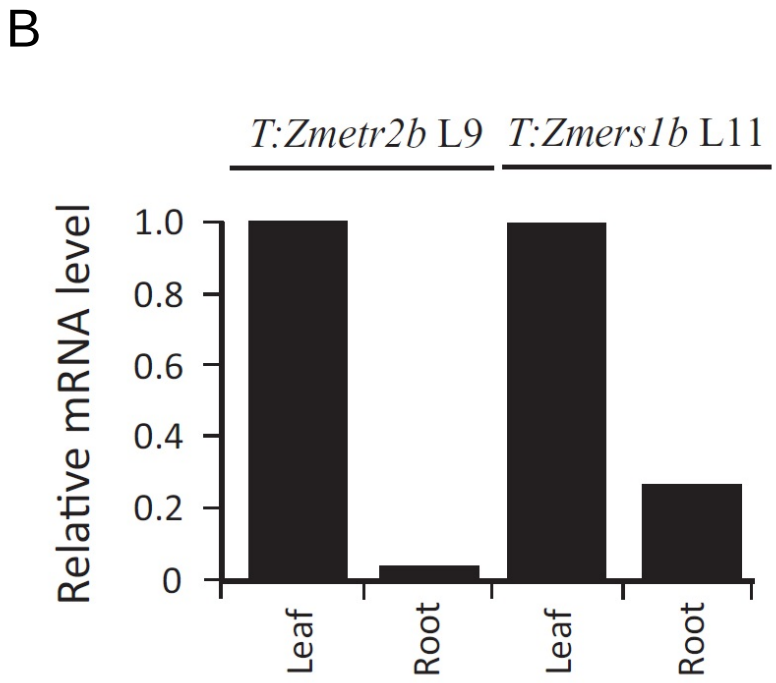

B
T:Zmetr2b L9
T:Zmers1b L11
Relative mRNA level
1.0
0.8
0.6
0.4
0.2
0
Leaf
Root
Leaf
Root

Figure 3.3. Arabidopsis expressing *Zmetr2b* or *Zmers1b* exhibit a larger leaf size and a delay in senescence. The same lines used in Figure 1 were grown under 100 µmol m-2 s-1 for 4 (A) or 7 (B) weeks. Wild-type (WT) plants were included as an ethylene sensitive control. *ein2-5* and *etr1-1* were included as ethylene insensitive controls. In (C), the size of leaf 6 from 4 week-old plants is compared for each line.

A


WT
ein2-5
etr1-1
L4
L5
L9
L11
L15
L12

B


WT
ein2-5
etr1-1
L4
L5
L9
L11
L15
L12

C

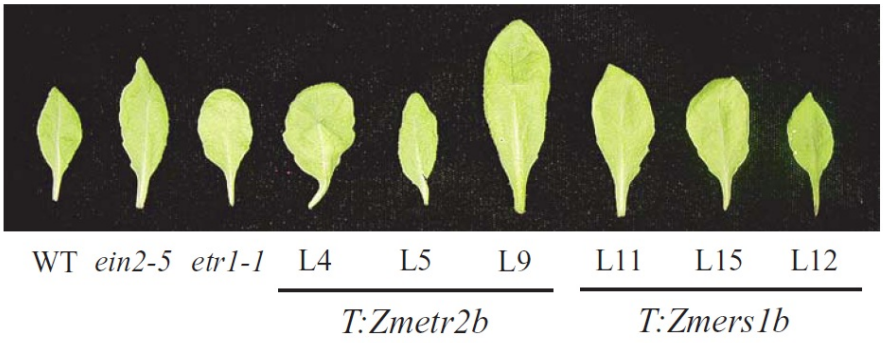
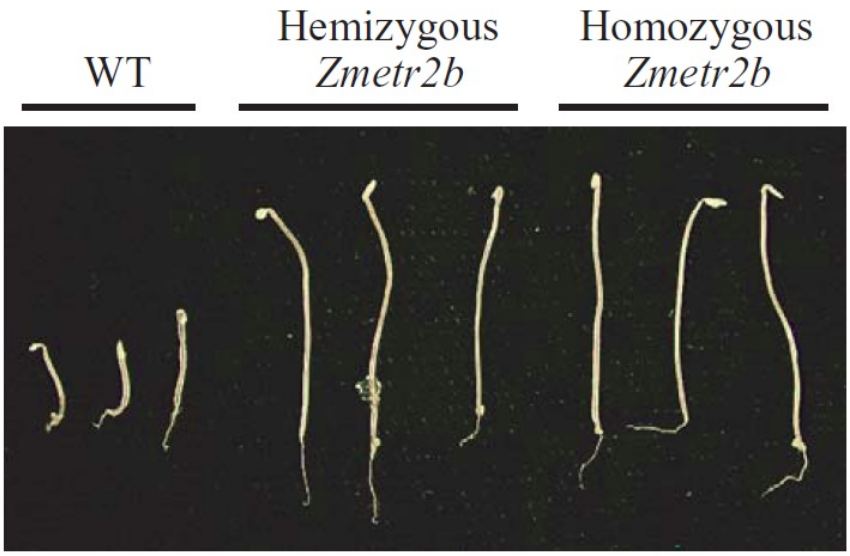

WT
ein2-5
etr1-1
L4
L5
L9
L11
L15
L12
T:Zmetr2b
T:Zmers1b

Figure 3.4. *Zmetr2b* and *Zmers1b* exert dominance in Arabidopsis in a hemizygous state. Line *T:Zmetr2b* L9 (A) and *T:Zmers1b* L11 (B) were crossed with wild-type (WT) Arabidopsis to generate seed hemizygous for each transgene. Seed containing each transgene in a hemizygous or homozygous state were germinated in the dark for 5 days on media with 20 μM ACC to assay for their triple response. Three representative seedlings are shown for each line. Quantitative measurements for hypocotyl and root lengths with standard deviations are shown in Table 3.3. Wild-type (WT) plants were included as an ethylene sensitive control.

A


WT
Hemizygous
Zmetr2b
Homozygous
Zmetr2b

B

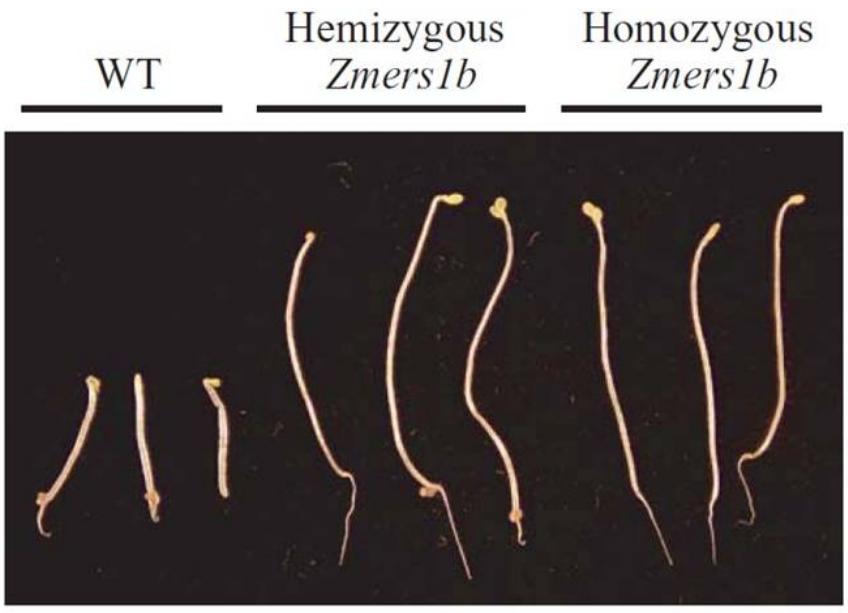

WT
Hemizygous
Zmers1b
Homozygous
Zmers1b

Figure 3.5. *Zmetr2b* and *Zmers1b* expression confers ethylene insensitivity over a range of ACC concentrations. Seeds of *T:Zmetr2b* L9 and *T:Zmers1b* L11 were germinated in the presence of either 5 μM $AgNO_3$ or ACC at the concentrations indicated and grown in the dark for 5 days. Wild-type (WT) plants were included as an ethylene sensitive control. Two representative seedlings are shown for each line.

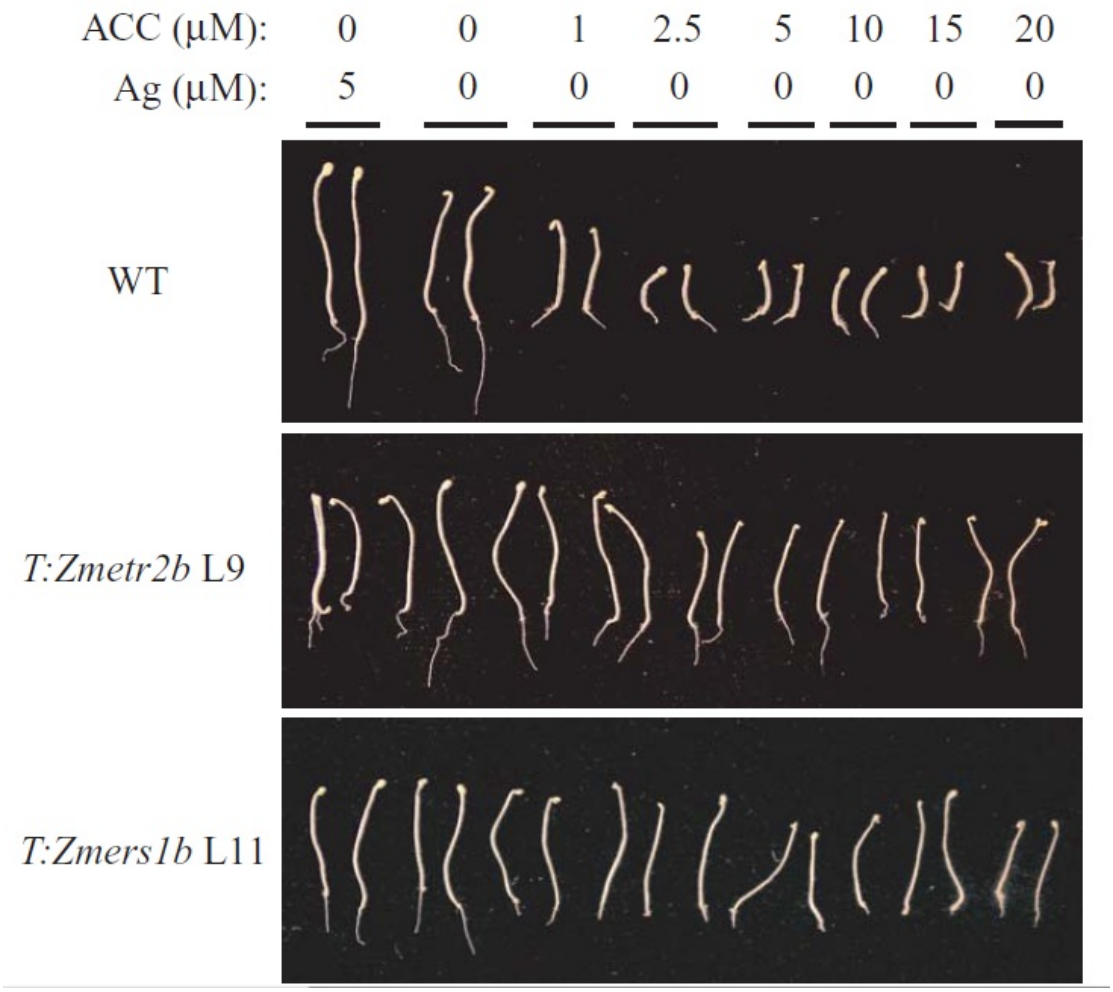
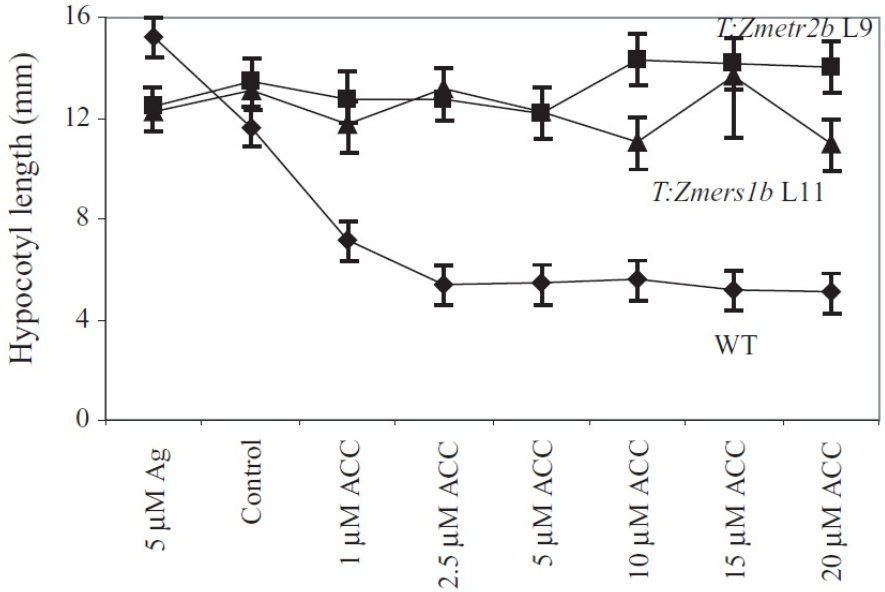

ACC (µM): 0 0 1 2.5 5 10 15 20
Ag (µM): 5 0 0 0 0 0 0 0
WT
T:Zmetr2b L9
T:Zmers1b L11

Figure 3.6. Quantitative measurement of the dominance of *Zmetr2b* and *Zmers1b* expression in Arabidopsis. Measurements were made of hypocotyl (A) and root (B) lengths of the *T:Zmetr2b* L9 (squares), *T:Zmers1b* L11 (triangles), and WT (diamonds) seedlings presented in Figure3. 5.

A


Hypocotyl length (mm)
16
12
8
4
0
T:Zmetr2b L9
T:Zmers1b L11
WT
5 µM Ag
Control
1 µM ACC
2.5 µM ACC
5 µM ACC
10 µM ACC
15 µM ACC
20 µM ACC

B

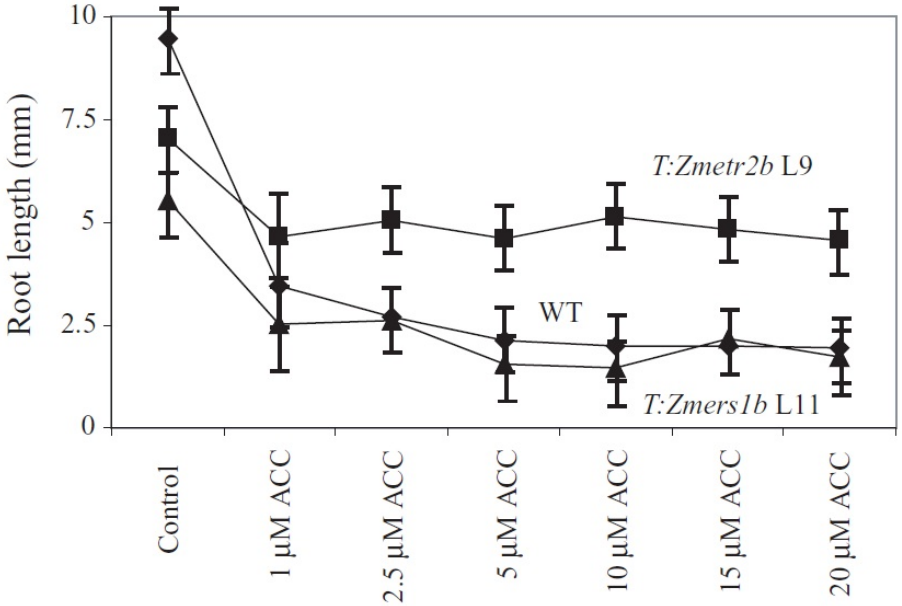

Root length (mm)
10
7.5
5
2.5
0
T:Zmetr2b L9
WT
T:Zmers1b L11
Control
1 µM ACC
2.5 µM ACC
5 µM ACC
10 µM ACC
15 µM ACC
20 µM ACC

Figure 3.7. Induction of ethylene regulated gene expression in Arabidopsis expressing *Zmetr2b* or *Zmers1b*. Northern analysis was performed on total RNA from light-grown *T:Zmetr2b* line L9 and *T:Zmers1b* line L11 plants that were treated either with 100 ppm ethylene (E) or air (A) for 24 hr. Following resolution and transfer of the RNA to membrane, the membrane was probed for the presence of *Zmetr2b* or *Zmers1b* mRNA using a combination of both probes (top panel). Northern analysis was also performed for *chiB* (basic chitinase) or *PDF1.2* (plant defensin 1.2) mRNAs which are ethylene regulated. Northern analysis was also performed for the translation elongation factor 1A (*eEF1A*) which served as an RNA loading control. Wild-type (WT) plants were included as an ethylene sensitive control. *ein2-5* and *etr1-1* were included as ethylene insensitive controls.

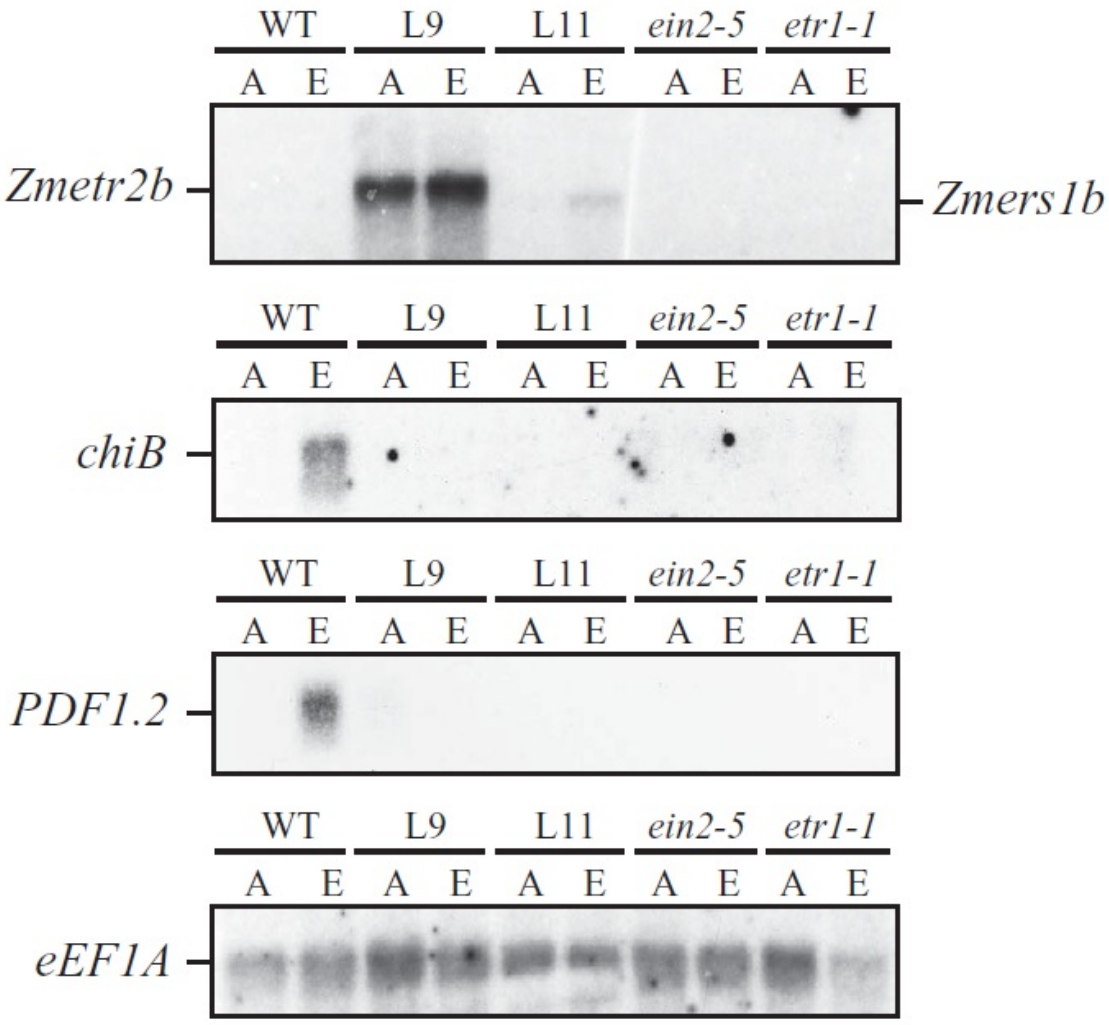
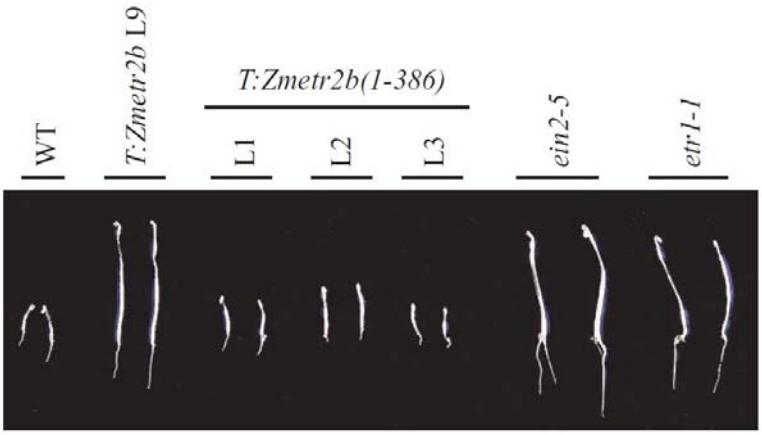
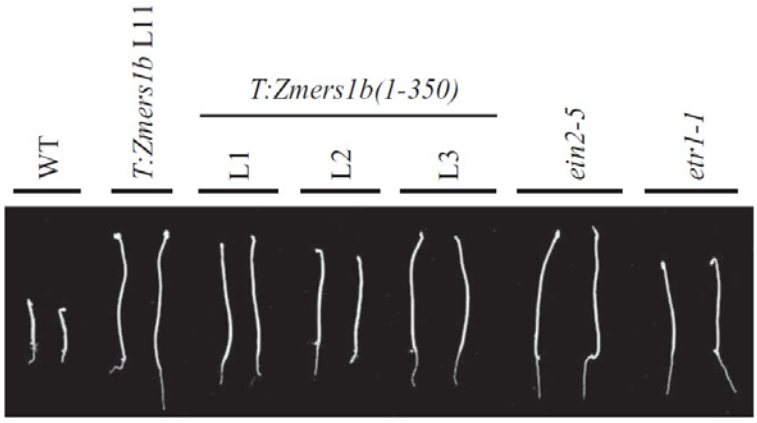

WT
L9
L11
ein2-5
etr1-1
A E A E A E A E A E
Zmetr2b
Zmers1b
WT
L9
L11
ein2-5
etr1-1
A E A E A E A E A E
chiB
WT
L9
L11
ein2-5
etr1-1
A E A E A E A E A E
PDF1.2
WT
L9
L11
ein2-5
etr1-1
A E A E A E A E A E
eEF1A

Figure 3.8. The N-terminal domain of *Zmers1b* but not *Zmetr2b* is sufficient to exert dominance in Arabidopsis. Arabidopsis was transformed with the portion of the *Zmetr2b* coding region representing the N-terminal domain, i.e., *Zmetr2b(1-386)*, to generate lines *T:Zmetr2b(1-386)* L1, L2, and L3 or transformed with the portion of the *Zmers1b* coding region representing the N-terminal domain, i.e., *Zmers1b(1-350)*, to generate lines *T:Zmers1b(1-350)* L1, L2, and L3. Seeds from lines homozygous for *Zmetr2b(1-386)* (A) or *Zmers1b(1-350)* (B) were germinated in the dark for 5 days on medium containing 20 μM ACC to assay for their triple response. *T:Zmetr2b* L9 and T*:Zmers1b* L11 were included as full-length controls. Wild-type (WT) plants were included as an ethylene sensitive control. *ein2-5* and *etr1-1* were included as ethylene insensitive controls. Two representative seedlings are shown for each line. Quantitative measurements for hypocotyl and root lengths with standard deviations are shown in Table 3.4. In (C), RNA was extracted from the same seedlings and Northern analysis performed to detect expression of full-length *Zmetr2b* or *Zmetr2b(1-386)* (left top panel), full-length *Zmers1b* or *Zmers1b(1-350)* (right top panel), or *eEF1A* (bottom panels) as an RNA loading control from the same membrane after it had been stripped.

A


WT
T:Zmetr2b L9
T:Zmetr2b(1-386)
L1
L2
L3
ein2-5
etr1-1

B


WT
T:Zmers1b L11
T:Zmers1b(1-350)
L1
L2
L3
ein2-5
etr1-1

C

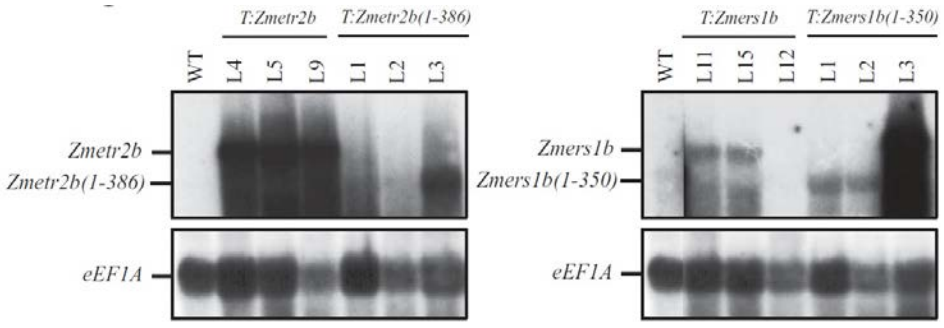

T:Zmetr2b
T:Zmetr2b(1-386)
WT
L4
L5
L9
L1
L2
L3
Zmetr2b
Zmetr2b(1-386)
eEF1A
T:Zmers1b
T:Zmers1b(1-350)
WT
L11
L15
L12
L1
L2
L3
Zmers1b
Zmers1b(1-350)
eEF1A

Figure 3.9. *Zmers1b* and *Zmetr2b* require expression of subfamily I receptors for their function. *T:Zmetr2b* line L9 or *T:Zmers1b* line L11 was crossed with *etr1-9;ers1-3/+* plants and F1 progeny that were *T:Zmetr2b;etr1-9/+;ers1-3/+* or *T:Zmers1b;etr1-9/+;ers1-3/+* were selfed. F2 progeny were germinated in the light for 2 weeks on medium. Examples of plants exhibiting the extremely small growth phenotype typical of *etr1-9;ers1-3* plants are shown and were genotyped by PCR analysis to determine the presence of each maize transgene as well as the presence of the *ETR1*, *etr1-9*, *ERS1*, and *ers1-3* loci as summarized in Table 3.5. Also shown are *etr1-9;ers1-3* and WT plants.

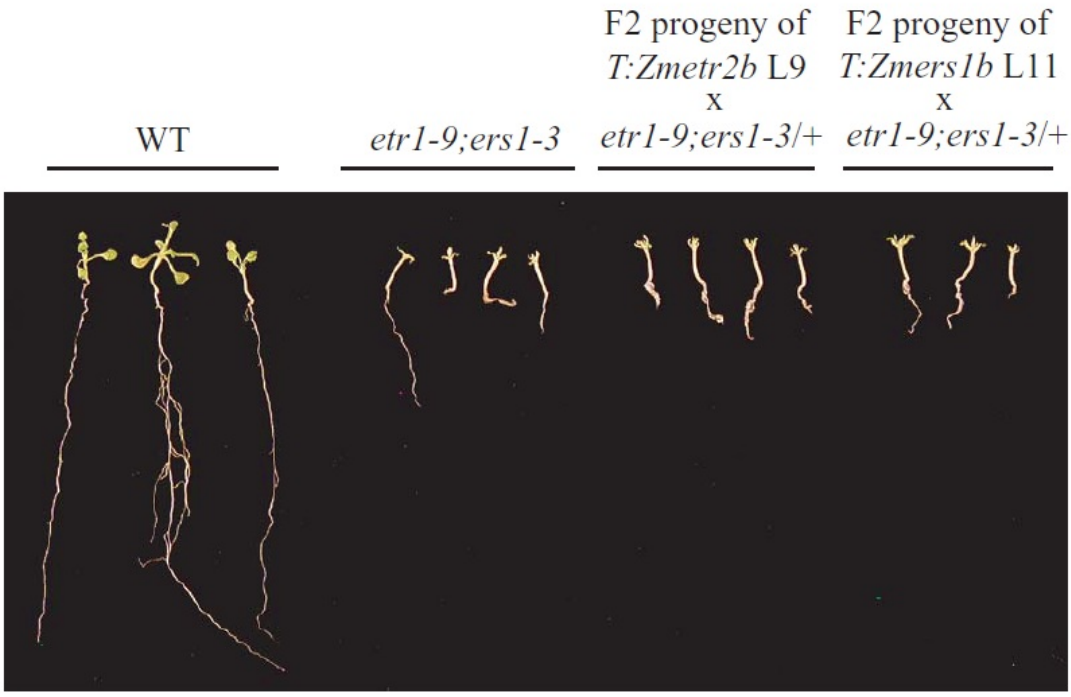

WT
etr1-9;ers1-3
F2 progeny of T:Zmetr2b L9 x etr1-9;ers1-3/+
F2 progeny of T:Zmers1b L11 x etr1-9;ers1-3/+

Table 3.1. Expression of mutant *Zmetr2* and *Zmers1* receptors confers ethylene insensitivity in Arabidopsis

	20 μM ACC		5 μM Ag^{2+}	
	Hypocotyl length (mm)[a] t-test	Root length (mm)[a] t-test	Hypocotyl length (mm)[a] t-test	Root length (mm)[a] t-test
WT	5.0 ± 0.71	3.00 ± 1.03	14.7 ± 1.12	6.69 ± 1.41
ein2-5	13.9 ± 1.71 P<0.001	7.43 ± 2.63 P<0.001	11.0 ± 3.13 P<0.001	6.27 ± 1.93 P=0.371
Zmetr2-4	14.2 ± 1.61 P<0.001	6.05 ± 1.36 P<0.001	13.0 ± 3.20 P<0.001	7.33 ± 1.81 P=0.122
Zmetr2-5	14.4 ± 1.97 P<0.001	4.87 ± 1.16 P<0.001	11.5 ± 2.87 P<0.001	5.39 ± 1.27 P<0.0001
Zmetr2-9	13.8 ± 2.36 P<0.001	6.27 ± 1.76 P<0.001	12.1 ± 2.40 P<0.001	7.07 ± 1.58 P=0.354
WT	6.6 ± 1.33	3.10 ± 0.92	16.9 ± 2.41	5.48 ± 0.94
ein2-5	17.0 ± 2.98 P<0.001	6.51 ± 1.56 P<0.001	14.7 ± 3.78 P<0.001	5.97 ± 1.56 P=0.349
Zmers1-11	17.6 ± 3.10 P<0.001	4.60 ± 0.88 P<0.001	10.9 ± 3.68 P<0.001	6.35 ± 1.87 P=0.086
Zmers1-15	18.0 ± 1.38 P<0.001	5.88 ± 1.40 P<0.001	11.7 ± 5.01 P<0.001	5.51 ± 1.45 P=0.921
Zmers2-12	8.8 ± 1.37 P<0.001	3.33 ± 0.97 P=0.406	17.3 ± 1.95 P=0.561	7.14 ± 1.97 P<0.001

[a]Measurements taken from 5 day old seedlings.

Table 3.2. Phenotypes of Arabidopsis transformants expressing maize *Zmetr2b* and *Zmers1b* receptors

	Flowering time[a] (days)	Leaf number	Chlorophyll a (ng/mg FW)	Chlorophyll b (ng/mg FW)	Chlorophyll a/b ratio
WT	22.0	11.1 ± 1.7	960 ± 92	281 ± 21	3.42
ein2-5	22.5	12.1 ± 2.0	923 ± 67	296 ± 21	3.11
T:Zmetr2b L4	22.5	12.9 ± 1.9	988 ± 86	298 ± 27	3.32
T:Zmetr2b L5	22.5	13.8 ± 2.0	903 ± 93	271 ± 16	3.33
T:Zmetr2b L9	28.0	16.2 ± 1.2	965 ± 104	304 ± 39	3.18
T:Zmers1b L11	24.0	12.5 ± 1.7	1016 ± 144	312 ± 33	3.25

[a]Measurements taken from plants grown under 100 μmol m^{-2} s^{-1}.

Table 3.3. *Zmetr2b* and *Zmers1b* are dominant when present in a hemizygous state in Arabidopsis

	Hypocotyl length (mm) [a]	t-test	Root length (mm) [a]	t-test
WT	6.37 ± 1.00		3.20 ± 1.14	
Hemizygous *Zmetr2b*	14.0 ± 2.04	P<0.001	6.01 ± 1.64	P<0.001
Homozygous *Zmetr2b*	17.2 ± 1.58	P<0.001	5.83 ± 1.18	P<0.001
WT	8.52 ± 0.65		2.78 ± 0.83	
Hemizygous *Zmers1b*	16.5 ± 4.98	P<0.001	4.78 ± 1.60	P<0.001
Homozygous *Zmers1b*	17.3 ± 2.74	P<0.001	4.77 ± 0.92	P<0.001

[a]Measurements for lines containing the *Zmetr2b* or *Zmers1b* transgenes were taken from plants grown for 4 or 5 days, respectively, on 20 μM ACC in the dark.

Table 3.4. Analysis of the ability of *Zmetr2b(1-386)* or *Zmers1b(1-350)* to confer ethylene insensitivity

	Hypocotyl length (mm) [a]	t-test	Root length (mm) [a]	t-test
WT	5.25 ± 0.39		2.81 ± 0.78	
ein2-5	16.0 ± 1.55	P<0.001	9.44 ± 1.66	P<0.001
etr1-1	15.0 ± 1.35	P<0.001	7.27 ± 1.83	P<0.001
T:Zmetr2b L9	16.6 ± 1.26	P<0.001	5.64 ± 1.27	P<0.001
T:Zmetr2b(1-386) L1	5.91 ± 0.83	P<0.001	2.66 ± 0.93	P=0.534
T:Zmetr2b(1-386) L2	6.17 ± 0.89	P<0.001	3.05 ± 0.91	P=0.321
T:Zmetr2b(1-386) L3	5.25 ± 0.59	P=0.977	2.84 ± 0.83	P=0.889
T:Zmers1b L11	19.4 ± 1.86	P<0.001	4.82 ± 1.56	P<0.001
T:Zmers1b(1-350) L1	16.4 ± 1.31	P<0.001	3.63 ± 0.97	P<0.005
T:Zmers1b(1-350) L2	12.4 ± 0.64	P<0.001	3.07 ± 0.56	P<0.05
T:Zmers1b(1-350) L3	18.6 ± 0.84	P<0.001	5.51 ± 0.86	P<0.001

[a]Measurements were taken from 4 day old seedlings grown on 20 μM ACC in the dark.

Table 3.5. *Zmetr2b* and *Zmers1b* require subfamily 1 receptors to confer ethylene insensitivity in Arabidopsis

Genotype	Number of small F2 progeny with or without *Zmetr2b* from a *T:Zmetr2b* L9 x *etr1-9;ers1-3*/+ cross		Number of small F2 progeny with or without *Zmers1b* from a *T:Zmers1b* L11 x *etr1-9;ers1-3*/+ cross	
	- *Zmetr2b*	+ *Zmetr2b*	- *Zmers1b*	+ *Zmers1b*
etr1-9/etr1-9;ers1-3/ers1-3	5	11	0	7
etr1-9/etr1-9;ERS1/ers1-3	1	1	1	0
etr1-9/etr1-9;ERS1/ERS1	0	1	0	0
ETR1/etr1-9;ers1-3/ers1-3	0	1	0	0
ETR1/etr1-9;ERS1/ers1-3	1	1	0	2
ETR1/etr1-9;ERS1/ERS1	0	0	0	0
ETR1/ETR1;ers1-3/ers1-3	0	0	0	0
ETR1/ETR1;ERS1/ers1-3	0	0	0	0
ETR1/ETR1;ERS1/ERS1	0	0	0	0

CHAPTER 4

Characterization of Ethylene-Mediated Gene Responses in Maize

ABSTRACT

Ethylene-regulated gene expression in seedlings of Zea mays was investigated using RT-PCR, Southern, and Northern analysis. Comparing expression profiles of several genes, e.g. ethylene receptor, ACC oxidase, of ethylene-treated and untreated wild-type B73 seedlings identified an ACC oxidase gene, *ZmACO35*, as an ethylene inducible gene in maize. The expression of *ZmACO35* showed different levels and patterns of induction in light-grown and etiolated seedlings. The maximum induction *of ZmACO35* is between 7 to 14 hours of ethylene treatment in light-grown seedlings and after 28 hours in dark-grown seedlings.

INTRODUCTION

Several genes are known to be induced by ethylene in plants, including those encoding ethylene receptors, ACC synthase (ACS), ACC oxidase (ACO), basic chitinase, ethylene-responsive factor (ERF1), and defensin, PDF1.2 (1-9). Ethylene receptors, ERS1, ERS2, and ETR2, are up-regulated in Arabidopsis leaves by ethylene (1). ACS and ACO together contribute to the positive feedback loop where ethylene treatment results in increased ethylene production.

ACS1 in Phalaenopsis and ACO2 in Arabidopsis are known to be induced by ethylene (2-5). Basic chitinase functions to degrade fungal cell wall chitin. In Arabidopsis, exposure of plants to ethylene induced high levels of systemic expression of basic chitinase with expression increasing with plant age (6,7). ERF1 is an immediate target of EIN3, and the expression of ERF1 can be activated rapidly by ethylene (8). PDF1.2, a defensin gene activated by pathogens, is induced by ethylene in Arabidopsis (9).

In maize, two types of ethylene receptors have been reported: one with homology to Arabidopsis ERS1 and a second that is the likely homolog of Arabidopsis ETR2 (10). The *ACS* and *ACO* gene families are considerably smaller in maize, with just three members, *ZmACS2*, *ZmACS6*, and *ZmACS7*,comprising the *ZmACS* family and four members, *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35*, comprising the *ZmACO* family (10). The four members of *ZmACO*s can be grouped into two subfamilies, i.e., *ZmACO20/ZmACO35* and *ZmACO15/ZmACO31*. ZmACO20 and ZmACO35 are highly similar in amino acid sequence (91% amino acid identity) and in the number and position of their introns. ZmACO15 and ZmACO31 are also highly similar to each other (96% amino acid identity).

An ethylene-responsive factor-like protein 1 (*ERF1*, AY672654) has been reported in maize. This maize ERF1-like protein shares 18% amino acid identity with Arabidopsis ERF1 (*AtERF1*, NM_113225) and no further function of that

protein has been addressed. A Zea mays cDNA clone (EE045700) shares 50% nucleic acid identity with *AtERF1* and its predicted amino acid sequence shares 36% amino acid identity. No basic chitinase has been reported in maize, but a Zea mays clone (EE176908) shares 61% nucleic acid identity with Arabidopsis basic chitinase (*AtChiB*, NM_112085.2) and its predicted amino acid sequence shares 28% amino acid identity.

To date, no gene has been reported as a marker gene of ethylene response in maize. In this study, the regulation of expression of maize gene encoding components of the ethylene biosynthetic and signaling machinery were investigated in ethylene-treated maize seedlings. Although *ERF1* and *basic chitinase* have not been identified in maize previously, the regulation of expression of maize clones, EE045700 and EE176908, were investigated as well. The levels of the induction of maize ACC oxidases were measured by semi-quantitative RT-PCR followed by Southern blot assay. To examine the regulation of the induction by light, the regulation of expression of maize ACC oxidases were compared in green and etiolated seedlings.

METARIAL AND METHODS

Reverse transcription polymerase chain reaction (RT-PCR)

.Seedlings were ground in liquid nitrogen, and total RNA was extracted with Qiagen RNeasy Plant Mini Kit. The first strand cDNA was made with Qiagen Omniscript Reverse Transcriptase, and genes were amplified by PCR with designed primers. PCR amplification was performed in 20 µl reactions containing 1 x PCR buffer, 0.4 u HotStarTaq DNA polymerase (Qiagen Inc, Valencia CA, USA), 250 µM dNTPs, 10 µM forward and reverse primers, and 100 ng cDNA. Reactions were carried out using the following conditions: 95ºC/5 min (1 cycle); 95ºC/30 sec, 55°C/30 sec, 72ºC/1 min (23-40 cycles varied by different genes); and a final extension at 72ºC/5 min (1 cycle)

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ZmACO35	ACGTCGACGACCGCTACAGGCAGGTG	AAGCCTGCAGCGGAAGGCAGTCTTCTC
ZmACO15	GGGGCACCATCAAGGACGCCTTCTCC	AATTATAATGCATTTAATAATTGGTCCCCTCCA
ZmACO31	GGGGCACCATCAAGGACGCCTTCTCC	TTTTAATAATGCATTCAATAATTGTTTTCCCTG
ZmACO20	GACGTCGACGACCACTACCGGCAAGTG	CGAAGGGAGAACACGGCAGTATTATGTGC
ZmBC	TTCTCTCACGATCACGATGATGAGAGC	GTAGTTGTAGTTGTAGGATATCTGGATGG
ZmETR2	ATGGTGGTGGGAACGGCGCCGTGCGGGG	TGCAGTCTGGAAGGAATTCCGAGCTTCC
ZmERS1	ATGGACGGATGTGATTGCATCGA	AACAGCTAGAAAATCATTGCGAGCACG
ZmERF1	GACATCGACGCATCCCATATCTATAGG	GCGCTCCACGGGAAAGTTGAGCACG
eIF4A	CATGCCCCCTGAGGCCCTTGAG	AGCAGGTCGGTGGTGATGAGCAC

Southern blot analysis

After PCR, DNA was resolved on a 1% agarose gel and transferred onto nylon membrane. PCR-generated *ZmACO15/31* and *ZmACO20/35* fragments were radiolabeled with dCTP using Prime-a-Gene labeling system (Promega, Madison WI, USA) and used for hybridization with the membrane overnight at 38°C in 5× SSPE (150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA), 5× Denhardt's solution, 50% formamide, and 1.5% SDS. Blots were washed for 30 min at 45°C in 1× SSPE/0.1% SDS, 30 min at 50°C in 0.5 × SSPE/0.1% SDS, and 30 min at 55°C in 0.2 × SSPE/0.1% SDS. The membrane was then exposed to film at -80°C with an intensifier screen.

Northern blot analysis

RNA was extracted by quick-freezing plant material in liquid nitrogen, grounding it to a fine powder, and resuspending 100 mg of the material in 1 ml TRIZOL® Reagent (Invitrogen, Carlsbad CA, USA). Following centrifugation, the supernatant was extracted with 200 µl chloroform and centrifuged to separate the phases. The RNA was precipitated from the aqueous phase by isopropyl alcohol, washed with 75% ethanol, resuspended in RNase-free H_2O, and resolved on a 1.2% agarose-formaldehyde gel and transferred onto nylon membrane. PCR-generated Zm*ACO15/31* and *ZmACO20/35* fragments were radiolabeled with dCTP using Prime-a-Gene labeling system (Promega, Madison WI, USA) and

used for hybridization with the membrane overnight at 38°C in 5× SSPE (150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA), 5× Denhardt's solution, 50% formamide, and 1.5% SDS. Blots were washed for 30 min at 45°C in 1× SSPE/0.1% SDS, 30 min at 50°C in 0.5 × SSPE/0.1% SDS, and 30 min at 55°C in 0.2 × SSPE/0.1% SDS. The membrane was then exposed to film at -80°C with an intensifier screen. Each Northern was repeated at least twice. The same membrane was stripped in 50% formamide, 2 × SSPE at 65°C for 30-60 min until no signal could be detected. Where indicated, the membrane was reprobed for α-tubulin mRNA using similar conditions.

RESULTS

An ACC oxidase, ZmACO35, is up-regulated by ethylene in maize

Maize B73 seed were germinated and grown in green house for 16 and 20 days, and six seedlings were used for each ethylene treatment in a chamber for 24 hours. A dose response of ethylene treatment was set up as: 1ppm, 10ppm, 25ppm, and 100ppm. As a control, six seedlings were grown under the same conditions but treated with air for 24 hours instead of ethylene. Seedlings were also treated with 1-MCP, an ethylene response inhibitor, twice for 1 hour each, during a 24-hour period.

The result of RT-PCR revealed an induction of *ZmACO35* to a level as low as 1 ppm of ethylene, and the response followed the increase of ethylene concentration (Fig. 4.1). Surprisingly, the ethylene inhibitor, 1-MCP, also induced the expression of *ZmACO35* (Fig.4.1). Other members of ACC oxidase gene family, i.e., *ZmACO15* and *ZmACO31*, did not show any significant response to ethylene treatment. The expression of ethylene receptors, *ZmETR2* and *ZmERS1*, showed a slight induction by ethylene. Similar results were also seen for the maize cDNAs, EE045700 and EE176908. The expression of *ZmeIF4A* served as an internal control and was unaffected by ethylene.

Expression of ZmACO35 exhibits a two-fold induction by exposure to saturating levels of ethylene

Maize B73 seed were germinated and grown in light for 15 days and treated with 100 ppm ethylene for 24 hours. Control seedlings were grown under the same conditions but treated with air for 24 hours instead of ethylene. The expression of maize ACC oxidases was investigated by semi-quantitative RT-PCR followed by Southern blot analysis. In order to quantitate changes in expression, the PCR reaction was performed for different numbers of cycles of amplification. For *ZmACO15*, *ZmACO31*, and *ZmACO35*, 23, 24, and 25 cycles were used, while 32, 33, and 34 cycles were used for *ZmACO20*. Ethylene treatment resulted in a more than two-fold increase in expression of *ZmACO20*

and *ZmACO35* (Fig.4.2). The expression of *ZmACO31* increased slightly whereas no significant response was observed for *ZmACO15* (Fig.4.2).

Induction of AmZCO35 by ethylene is light-regulated

Maize B73 seed were germinated in the light or in the dark for nine days and treated with air or 100 ppm ethylene for 7, 14, 28 or 40 hours. A control group of seedlings were treated with 1-MCP for 28 hours. The expression of maize ACC oxidases, *ZmACO20/35* and *ZmACO15/31*, was investigated by Northern analysis. The result showed different levels and patterns of induction of *ZmACO35* between light and dark-grown seedlings.

The level of induction of *ZmACO35* was greater in etiolated seedlings than in light-grown seedlings. Analysis of 40 µg RNA was sufficient to detect the expression of *ZmACO35* in etiolated seedlings (Fig.4.3.B), while 100 µg RNA was needed in light-grown seedlings (Fig.4.3.C.). The maximum induction of *ZmACO35* is at, or following, 28 hours in etiolated seedlings (Fig.4.3.B), and between 7 to 14 hours of ethylene treatment in light-grown seedlings (Fig.4.3.C). A slight repression of *ZmACO15/31* expression was observed in etiolated seedlings (Fig.4.3.B), but no significant induction of *ZmACO15/31* expression was observed in light-grown seedlings (Fig.4.3.C).

DISCUSSION

Four ACC oxidases (i.e., *ZmACO15*, *ZmACO20*, *ZmACO31*, *ZmACO35*), two ethylene receptors (i.e., *ZmETR2*, *ZmERS1*), and two maize clones (i.e., EE045700, EE176908) were examined for their responsiveness to ethylene. Among the four ACC oxidases, only *ZmACO35* showed substantial induction by ethylene. In Arabidopsis, the *ACO* gene family may be composed of up to 17 members although not all may function as ACC oxidases (11). Only specific members of this family, e.g. *ACO2*, are regulated by ethylene (4,5). This similarity indicates the existence of differential regulation of ACC oxidase isoforms in both maize and Arabidopsis.

Genes encoding ERF1 or basic chitinase have not been characterized in either maize or other monocots, e.g. rice. Therefore, primers for PCR were designed based on sequences conserved between the putative maize homologs and Arabidopsis, i.e., between EE045700 and *AtERF1* and between EE176908 and *AtChiB*. The result of RT-PCR analysis revealed small levels of induction of EE045700 and EE176908 to ethylene. The lower level of induction for these putative homologs may indicate differences in regulation in maize relative Arabidopsis. Another possibility is the putative maize *ERF*1 and *ChiB* are not targets of ethylene signaling.

In the endosperm, the level of expression from *ZmACO31* and *ZmACO35* in the embryo was several orders of magnitude lower than that of *ZmACO20* (12).

However, in this study, the induction of *ZmACO20* was not detected solely by RT-PCR (data not shown), but was only revealed by RT-PCR followed by Southern blot analysis. Under the same condition, PCR for *ZmACO20* required seven additional amplification cycles for detection than for *ZmACO35*. This result indicates at least 100-fold lower expression of *ZmACO20* in young seedlings.

ZmACO20 and *ZmACO35* share 90% nucleotide identity but differ in size by 330 base pairs. Therefore, although both genes can be detected by the same probe in Northern analysis, they are distinguishable in size. In contrast, the mRNA sequences of *ZmACO15* and *ZmACO31* share 95% identity and do not differ in size. Therefore, Northern analysis of this subfamily represents the combinatorial expression from both genes. Expression from *ZmACO35* is induced by ethylene whereas that of *ZmACO15/31* is slightly repressed in etiolated seedlings. In the absence of exogenous ethylene, both *ZmACO35* and *ZmACO15/31* had higher basal expression in etiolated seedlings than in light-grown seedlings. Etiolated seedlings may experience more ethylene due to a higher endogenous ethylene production which may be sufficient for maximal induction of expression from the *ZmACO15/31* subfamily and additional exogenous ethylene may exceed this level resulting in the repression of expression from the *ZmACO15/31* subfamily.

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Figure 4.1. *ZmACO35* is up-regulated by ethylene. Seven genes were tested in 16- and 20-day old light-grown maize seedlings for ethylene responsiveness after a 24-hour ethylene treatment. *ZmACO35* can be better induced by the ethylene treatment. Two other ACC oxidase genes (*ZmACO15* and *ZmACO31*) and two ethylene receptor genes (*ZmETR2* and *ZmERS1*) are not very responsive to ethylene. Two putative genes (EE045700 for ethylene-responsive factor, *ZmERF1*, and EE176908 for basic chitinase, *ZmBC*) are not well induced, either. The translation initiation factor, *eIF4A*, serves as the internal control. Each sample represents a pool of 6 seedlings.

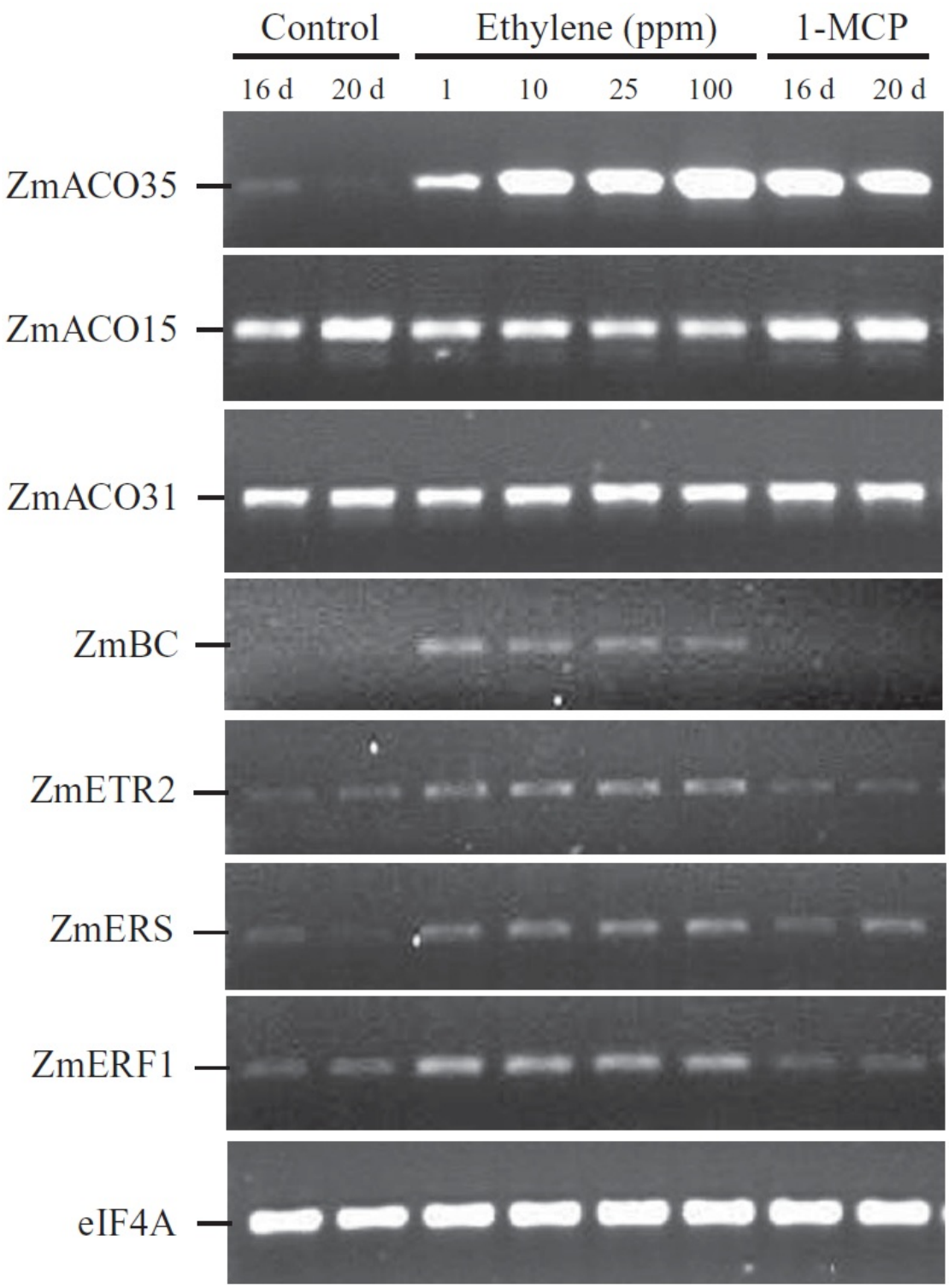

Control
Ethylene (ppm)
1-MCP
16 d
20 d
1
10
25
100
16 d
20 d
ZmACO35
ZmACO15
ZmACO31
ZmBC
ZmETR2
ZmERS
ZmERF1
eIF4A

Figure 4.2. The induction of *ZmACO35* by 100ppm ethylene. The RT-PCR followed by Southern blot analysis showed more than two-fold increases on the expression levels of *ZmACO20* and *ZmACO35* by ethylene treatment. The expressions of *ZmACO15* and *ZmACO31* did not show much responsiveness to ethylene. The PCR was performed with three adjacent numbers of amplifying cycles: 23, 24, and 25 cycles for *ZmACO15*, *ZmACO31*, and *ZmACO35*, and 32, 33, and 34 cycles for *ZmACO20*.

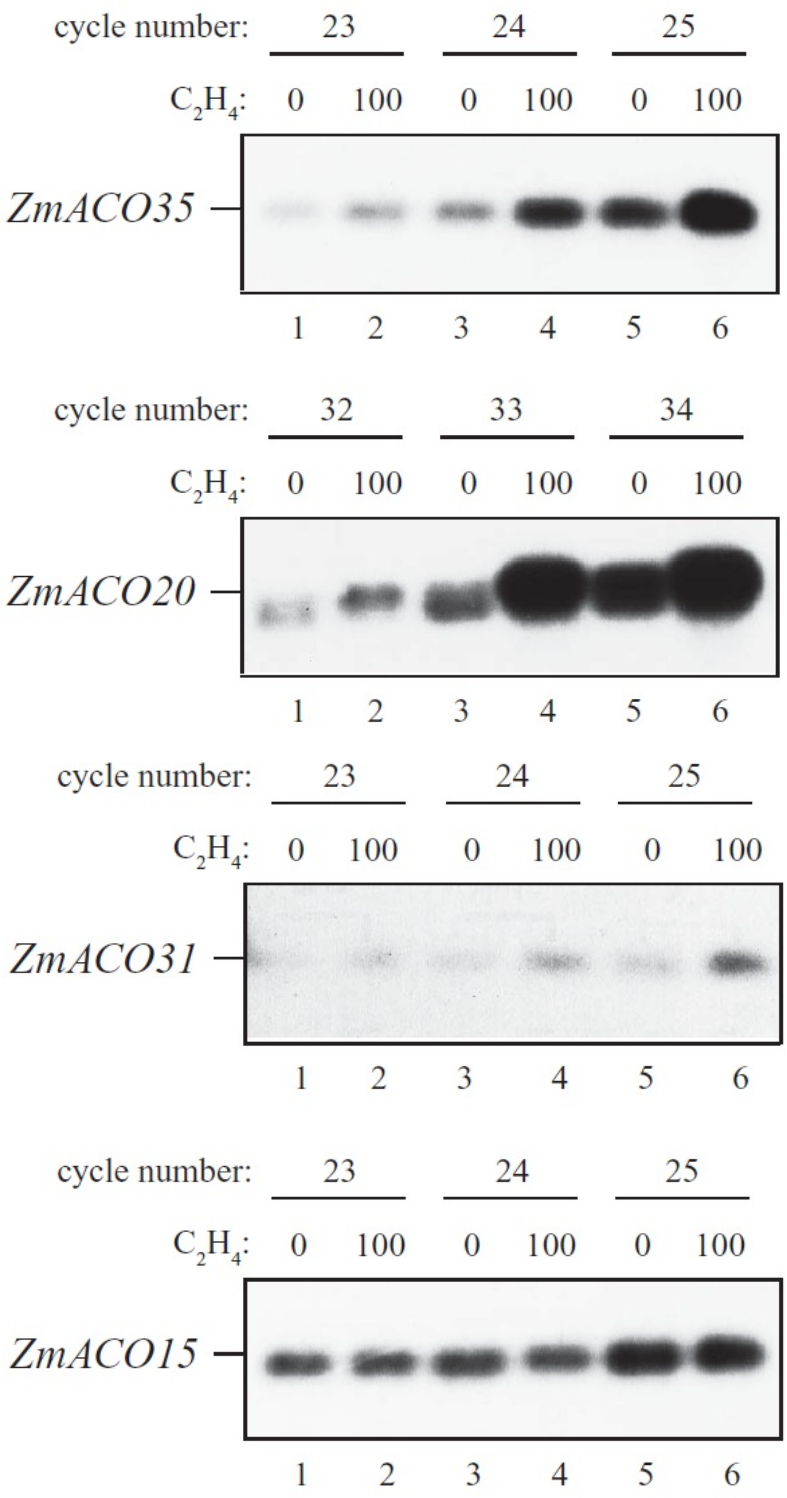
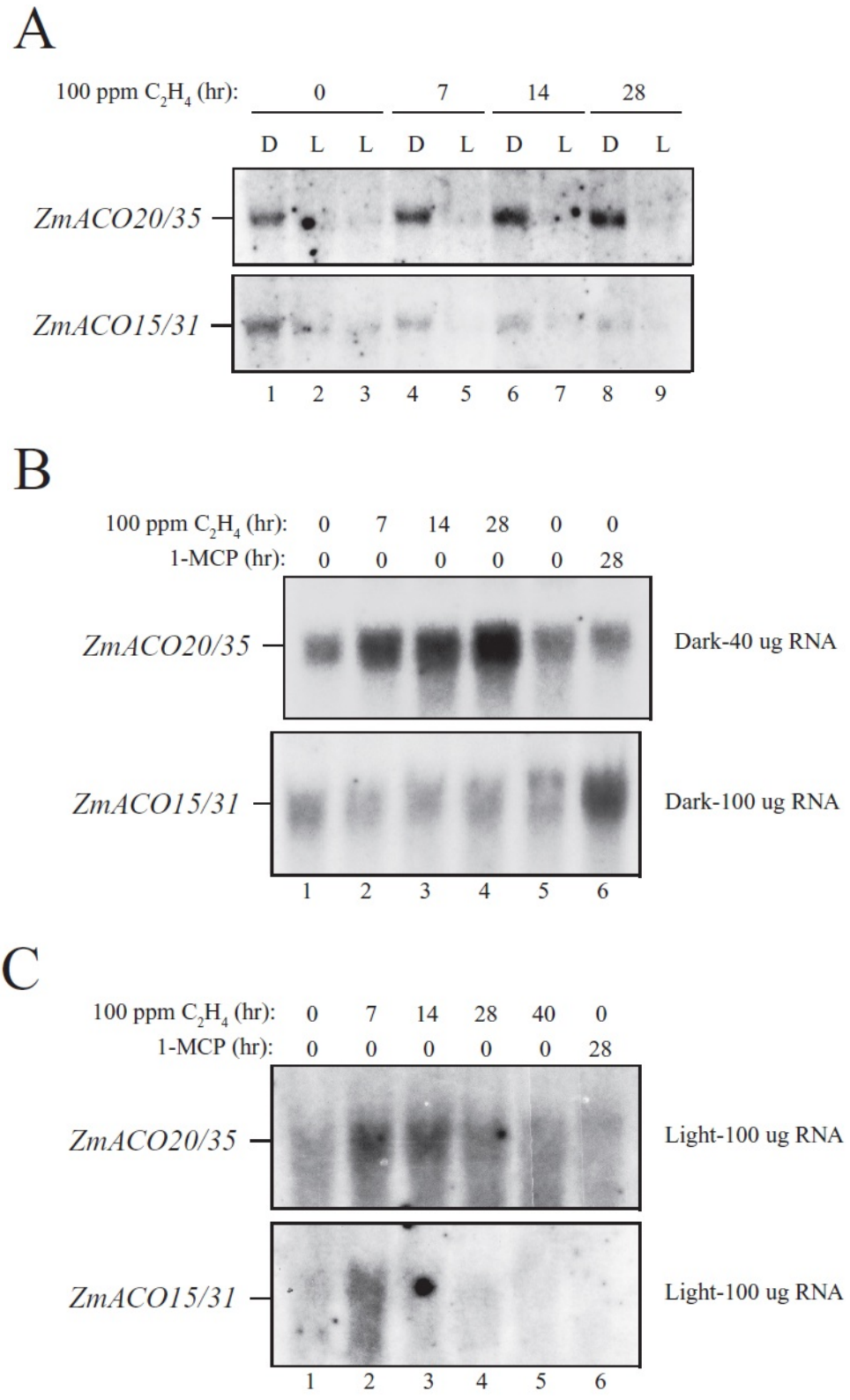

cycle number: 23 24 25
C2H4: 0 100 0 100 0 100
ZmACO35
1 2 3 4 5 6
cycle number: 32 33 34
C2H4: 0 100 0 100 0 100
ZmACO20
1 2 3 4 5 6
cycle number: 23 24 25
C2H4: 0 100 0 100 0 100
ZmACO31
1 2 3 4 5 6
cycle number: 23 24 25
C2H4: 0 100 0 100 0 100
ZmACO15
1 2 3 4 5 6

Figure 4.3. The induction of *ZmACO35* in green and etiolated seedlings. (A) Light-grown and dark-grown maize seedlings were treated without or with 100ppm ethylene for 7, 14, or 28 hours. The level of induction of *ZmACO35* is greater in etiolated seedlings than in green seedlings. The expression of *ZmACO15/31* is slight repressed by ethylene in etiolated seedlings. 40µg of total RNA was loaded. (B) Dark-grown maize seedlings were treated without or with 100ppm ethylene for 7, 14, or 28 hours. A group of seedlings were treated with 1-MCP for 28 hours. A loading of 40 µg RNA is sufficient to detect the induction of *ZmACO35* while 100µg of RNA were loaded for the expression of *ZmACO15/31*. The maximum induction of *ZmACO35* is at or after 28 hours of ethylene treatment. (C) Light-grown maize seedlings were treated without or with 100ppm ethylene for 7, 14, 28 or 40 hours. A group of seedlings were treated with 1-MCP for 28 hours. The maximum induction of *ZmACO35* is between 7 to 14 hours of ethylene treatment. 100µg of total RNA was loaded.


A
100 ppm C_2H_4 (hr): 0 7 14 28
D L L D L D L D L
ZmACO20/35
ZmACO15/31
1 2 3 4 5 6 7 8 9
B
100 ppm C_2H_4 (hr): 0 7 14 28 0 0
1-MCP (hr): 0 0 0 0 0 28
ZmACO20/35
Dark-40 ug RNA
ZmACO15/31
Dark-100 ug RNA
1 2 3 4 5 6
C
100 ppm C_2H_4 (hr): 0 7 14 28 40 0
1-MCP (hr): 0 0 0 0 0 28
ZmACO20/35
Light-100 ug RNA
ZmACO15/31
Light-100 ug RNA
1 2 3 4 5 6

CHAPTER 5.

Tissue-Specific Expression of a Dominant-Negative Ethylene Receptor Gene in Maize

ABSTRACT

The plant hormone ethylene regulates diverse aspects of plant growth and development and regulates responses to adverse growth conditions. Although ethylene insensitive and hypersensitive mutants have been studied extensively in Arabidopsis to elucidate the roles of ethylene in plants, the approach to date has been the study of mutations that function at the whole-plant level. In such mutants, a phenotype observed in the flower, for example, may be influenced indirectly by effects on leaf or root growth and development. In order to study the effect of ethylene on specific organs, a dominant negative mutant maize ethylene receptor gene was designed to be expressed in a spatial or temporal specific manner. By placing the dominant negative mutant receptor gene, i.e. *Zmetr2*, under the control of organ-specific promoters, i.e. *RbcS-m3*, *PEPC*, or *Shrunken1*, a state of ethylene insensitivity can be restricted to that part of plant. Therefore, it is possible to examine the roles of ethylene in photosynthesis and leaf development; in responding to drought stress, or during endosperm cell death and kernel abortion in maize. Furthermore, it allows the study of the roles of ethylene in different situations, e.g., how maize signals an environmentally-

induced stressed state through ethylene and when or in what organs maize responds to ethylene-mediated stresses.

INTRODUCTION

Ethylene, which is produced by almost all plants, mediates a wide range of different plant responses and developmental steps. Ethylene plays an active role in seed germination, tissue differentiation, formation of root and shoot primordia, root elongation, lateral bud development, flowering initiation, anthocyanin biosynthesis, flower opening and senescence, pollination, fruit degreening and ripening, the production of volatile organic compounds responsible for aroma formation in fruits, leaf and fruit abscission, the response to biotic and abiotic stress, and plant-microbial interactions that are important for the growth and survival of a plant (1-3).

Leaf expansion is the result of cell division and cell expansion. The total leaf area of rosette leaves of ethylene-insensitive Arabidopsis mutants (etr1-1 and ers1) was reported to be 25–50% larger than those of wild-type plants (4, 5). The increase in total leaf area was attributed to increased cell expansion in ethylene insensitive plants (5). In contrast with the finding that ethylene treatment inhibits elongation growth are reports that low concentrations (i.e., below 0.1 ppm) can stimulate leaf expansion (6, 7), stem elongation (8-10), hypocotyl elongation (11), and root elongation (12). To explain these differential responses to ethylene, a

biphasic model was proposed (6, 12), in which low levels of ethylene promote and high levels inhibit cell expansion.

Photosynthesis is a process that converts carbon dioxide into organic compounds, especially sugars, using the energy from sunlight. Ethylene was found to affect this process. Stomatal conductance was lower in ethylene-insensitive Arabidopsis mutants but higher in ethylene-insensitive tobacco when compared to ethylene-sensitive controls (3). This indicates that the effect of ethylene on stomatal regulation, and thus photosynthesis, may differ among species. In addition to effects on stomatal conductance, ethylene may play a role in the regulation of photosynthesis via its role in sugar sensing. Recent studies on the interaction between ethylene, abscisic acid (ABA), and sugar sensing suggest that ethylene may play a role in the regulation of photosynthetic gene expression (13). Ethylene-insensitive plants appear to be more sensitive to endogenous glucose levels, while the application of an ethylene precursor decreases a plant's sensitivity to glucose (14).

Leaf senescence is considered the last stage of leaf development and is a genetically programmed process that is highly regulated with recycling of reserves from the senescing leaves to other storage organs (seeds, trunk, branches). During early leaf development, ethylene production is high but declines when leaves reach the fully expanded stage only to increase again during senescence. The first evidence of ethylene as a promoter of leaf

senescence was observed in leaves and stems treated with ethylene. In particular, leaves exposed to ethylene show chlorosis (yellowing), necrosis (death), and shattering (drying and breaking). The first onset of ethylene-induced senescence in leaves involves a decrease in photosynthesis and loss of chlorophyll (15). However, the ethylene response is variable and depends on the species because every plant or part of a plant has different sensitivity to this hormone (3). In maize, ACC synthase (ACS) mutants were isolated and found to inhibit drought-induced senescence (16). The mutations affect the rate-limiting step in ethylene biosynthesis and these mutants were shown to have reduced ethylene synthesis, suggesting that ethylene may also mediate drought-induced senescence. It is still unclear how ethylene promotes senescence but it was found that ethylene could inhibit abscisic acid (ABA)-induced stomatal closure in Arabidopsis (17). Together, these observations indicate that ethylene may function in stomata opening, so that the lower ethylene produced in ACS mutants could confer greater drought tolerance. With increasing climate change occurring as a consequence of global warming, it will become important to understand how ethylene controls drought stress responses.

Most plants use the C3 pathway of photosynthesis to convert light energy into chemical energy. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes primary carbon fixation, in which a five-carbon sugar phosphate, ribulose-1, 5-bisphosphate, and CO_2 are converted to two molecules of the three-carbon compound, 3-phosphoglycerate. The C4 pathway is a

complex adaptation of the C3 pathway that overcomes the limitation of photorespiration. Maize, or corn, is an important economic cereal crop to human beings that utilizes the C4 pathway. In maize, CO_2 is initially fixed by the enzyme, phosphoenolpyruvate carboxylase (PEPC), to form the C4 acid oxaloacetate, which is reduced by NADPH from the light reaction to form malate. The fixed carbon in the form of malate is transported from the mesophyll to the bundle sheath chloroplasts where it is decarboxylated and the CO_2 released is fixed through the Calvin cycle. The three carbon compound pyruvate diffuses back to the mesophyll where it is phosphorylated by ATP to regenerate the carbon acceptor phosphoenolpyruvate (18). Given their important roles for photosynthesis activity in maize, Rubisco and PEPC are expressed to high levels in green leaves.

In maize, ethylene is involved in the abortion of the caryopsis and the sensitivity is crucial in this organ's response to the hormone (19). Kernels at 3 DAP are more sensitive to ACC than at 10 DAP (20), and kernels at the ear tip are developmentally about 4 to 6 days behind the basal kernels in normal ear development (21). Shading induces ACC and ethylene production in both apical and basal kernels whereas only apical kernels revealed abortion symptoms (19). Ethylene is a promoter of grain maturation and ear senescence. Application of ethylene inhibitors delayed grain maturation and increased final grain size implying that there is a potential for increasing grain yield in wheat and rice by delaying maturation through proper manipulation of ethylene synthesis or action

(22, 23). Cereal endosperm is composed of storage, transfer and aleurone cells, each of which has a distinctive structural and physiological role in the overall development of this storage tissue (24). The majority of endosperm tissue is composed of storage cells that synthesize starch and storage proteins as a reserve to support growth of the seedling following germination. In maize, endosperm cell death is a developmentally controlled process and ethylene is the signal responsible for mediating pleiotropic effects associated with sh2 kernel development and is involved in the signal transduction pathway leading to endosperm programmed cell death (25).

Starch production is critical to both the yield and the quality of the grain. In the maize endosperm, sucrose is converted to glucose and then into starch that normally accounts for 73% of the kernel's total weight. Plant genetic and biochemical approaches have so far identified over 20 genes involved in starch production (26-28). Shrunken 1 (Sh1), located upstream in the pathway, aids in the formation of glucose. High Sh1 activity plays a role in better grain filling, probably by providing more glucose for ADP-glucose pyrophosphorylase (AGPase) (29, 30). The *Sh1* gene is highly transcribed in the developing endosperm of the kernel (31). However, high levels of mRNA could be detected not only in the maize kernel, but also in the roots and shoots of etiolated young seedlings (32). In etiolated shoots, the transcript level is negatively affected by illumination. In addition to this developmentally controlled expression profile, the transcript level can be increased up to 20-fold in roots and, due to the low basal

level, up to 250-fold in young green leaves upon anaerobic stress (32). The organ-specific gene expression and the anaerobic stress response provide a model system to investigate transcriptional control of plant genes.

When kernels are developing, stress conditions perceived by leaves can communicate to the ears to induce kernel abortion at the ear tip and result in fewer kernels per ear. Stress conditions can also exist even under ”normal” growth conditions as the number and size of kernels per ear are a result of the nutritional and photosynthetic status of the leaves. Recently, two maize ethylene receptors, ZmETR2 and ZmERS1, were analyzed in Arabidopsis and the Cys to Tyr mutation in these receptors conferred ethylene insensitivity (33). In this study, the dominant negative mutant receptor gene, *Zmetr2*, was placed under the control of the organ-specific promoter, i.e. *RbcS*, *PEPC*, or *Sh1*, to determine the role of ethylene in leaf development and function, kernel development, and in stress responses.

MATERIALS AND MATHODS

Plasmid constructs and mutagenesis

For leaf-specific *Zmetr2* expression constructs, the DNA fragments of the promoter regions of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (*rbcS-m3*; U09743.1) and phosphoenolpyruvate

carboxylase (*PEPC*; X15239) were obtained by PCR from B73 maize genomic DNA. The oligonucleotides used were *ZmRbcS*-F1: 5'-CCGAATTCGAGCTCCCTTTAATCTGGCGCTAG-3'/ *ZmRbcS*-R1: 5'-GGCTCGAGTCCTGTAGTAGGCTCCCCCAC-3' and *ZmPEPC*-F1: 5'-GGGAATTCACTTTTTTTTTTCCTTATCCTCCTAGG-3'/ *ZmPEPC*-R1: 5'-GGCTCGAGATTGGTGATCAATGCAGTGCGC-3'. EcoR I and Xho I sites were used to replace the original CaMV 35S promoter with these leaf-specific promoters on the pFGC5941 vector. The cDNA of *ZmETR2* was obtained by RT (reverse transcriptase) reaction from B73 maize genomic DNA. The oligonucleotides used were ZmETR2-40-F1A: 5'-GCTCTAGACCATGGTGGTGGGAACGGCGCCGTGCGGG-3' and *ZmETR2-40*-R1A: 5'-GCAGATCTTTCAACTGTTCTGAAGGACCCTATAC-3'. The generation of *Zmetr2* mutant was performed using the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega, Madison WI, USA) as described previously (33), and the resulting *Zmetr2* gene was cloned in pBI121 vector by Xba I and Bgl II sites. An Xho I-Xba I-Sma I adaptor was used to replace the original CHSA intron on the pFGC5941 vector and subcloned the *Zmetr2* gene from pBI121 vector by Xba I and Sma I sites.

For the kernel-specific *Zmetr2* expression construct, the DNA fragment of the promoter region of the *Shrunken1* was released from pSHGUS vector and subcloned into the pFGC5941 vector by Xho I and Nco I sites after the original *CaMV 35S* promoter on the pFGC5941 was replaced by an EcoR I-Xho I adaptor.

The original CHSA intron on the pFGC5941 was replaced with *Zmetr2* mutant gene by Nco I and BamH I sites.

Plant material and transformation

Maize transformation was performed by the Plant Transformation Facility (Iowa State University) using particle bombardment of Hi II immature zygotic embryos. Stable transformation events were screened by PCR and selected for the presence of the selectable marker associated with the transgene. Transgenic plants were regenerated and acclimatized to soil in the greenhouse.

PCR Analysis

DNA was isolated by quick-freezing plant material in liquid nitrogen, grounding to a fine powder, and resuspending in 400 µl extraction buffer (100 mM Tris-Cl pH 9.0, 20 mM EDTA, 200 mM NaCl, 1 % Sarcosyl, and 1% β-ME). Following centrifugation, the supernatant was extracted with 400 µl phenol: chloroform (1:1) and centrifuged to separate the phases. The DNA was precipitated from the aqueous phase by sodium acetate and isopropyl alcohol, washed with 75% ethanol and resuspended in H_2O. PCR amplification was performed in 20 µl reactions containing 1 x PCR buffer, 0.4 u HotStarTaq DNA polymerase (Qiagen Inc, Valencia CA, USA), 250 µM dNTPs, 10 µM forward and

reverse primers, and 50 ng genomic DNA. Reactions were carried out using the following conditions: 95ºC/5 min (1 cycle); 95ºC/30 sec, 55°C/30 sec, 72ºC/1 min (35 cycles); and a final extension at 72ºC/5 min (1 cycle). To detect the presence of *Rubisco-Zmetr2*, the forward primer, *ZmRbcS*-F3, is 5'-GCACATCACGCATAGTCCAACCATGG-3', and the reverse primer, *ZmETR2*-R6, is 5'-GAGCAGGTGGCGAAGTAGAGCAGC-3'. To detect the presence of *PEPC-Zmetr2*, the forward primer, *ZmPEPC*-F4, is 5'-AACAGCAGCAAGCCAAGCCAAAAAGG-3', and the reverse primer is *ZmETR2*-R6. To detect the presence of *Shrunken1-Zmetr2*, the forward primer, *ZmSH*-F1, is 5'-GGCAACTGTTTTGCTATAAGATTCCATG-3', and the reverse primer is *ZmETR2*-R6.

Northern analysis

Plant material was quick-frozen in liquid nitrogen, and ground to a fine powder. For leaf and root, 100 mg sample powder was resuspended in 1 ml TRIZOL® Reagent (Invitrogen, Carlsbad CA, USA), and RNA was extracted as described previously (33).

For samples rich in polysaccharides, e.g. kernel and ovary, 100 mg sample powder was resuspended in 500 ml 65°C CTAB buffer (100 mM Tris pH 6.8, 25 mM EDTA pH 8.0, 2% CTAB, 2% PVP, 1.4M NaCl, 5% β-ME) and 375 µl chloroform. Following centrifugation, the supernatant was extracted with 350 µl

acid-phenol (non-buffered, non-saturated phenol with 35% guanidine thiocyanate), 45 µl 2 M sodium acetate, 250 µl chloroform and centrifuged to separate the phases. The supernatant was extracted with 250 µl chloroform again and centrifuged to separate the phases. The RNA was precipitated from the aqueous phase by isopropyl alcohol, washed with 75% ethanol, and resuspended in RNase-free H_2O.

For pollen, 100 mg sample powder was resuspended in 500 µl 50°C extraction buffer (300 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 2% SDS, 10mM β-ME), 70 µl 3M KCl, and incubated on ice for 20 min. Following centrifugation, the RNA was precipitated from the supernatant by 250 µl 8 M LiCl at 4°C overnight. The pellet was dissolved in 100 µl RNase-free H_2O, extracted with 200 µl phenol (saturated with 0.1 M Tris-HCl pH 8.0) and centrifuged to separate the phases. The RNA was precipitated again from the supernatant by 10 µl 5M NaCl and 250 µl ice-cold 90% ethanol at -70°C overnight, washed with 75% ethanol, and resuspended in RNase-free H_2O.

The RNA was resolved on a 1.2% agarose-formaldehyde gel. PCR-generated Zmetr2-OCS 3’ fragments were radiolabeled with dCTP using Prime-a-Gene labeling system (Promega, Madison WI, USA) and used for hybridization with the membrane overnight at 38°C in 5× SSPE (150 mM NaCl, 10 mM NaH2PO4·H2O, 1 mM EDTA), 5× Denhardt's solution, 50% formamide, and 1.5% SDS. Blots were washed for 30 min at 45°C in 1× SSPE/0.1% SDS, 30 min

at 50°C in 0.5 × SSPE/0.1% SDS, and 30 min at 55°C in 0.2 × SSPE/0.1% SDS. The membrane was then exposed to film at -80°C with an intensifier screen. Each Northern was repeated at least twice. The same membrane was stripped in 50% formamide, 2 × SSPE at 65°C for 30-60 min until no signal could be detected.

RESULTS

Expression from Zmetr2 is tissue-specific

To investigate the expression pattern of *Zmetr2* in transgenic plants, transformant lines homozygous for *Rubisco-Zmetr2* (*RbcS::Zmetr2*), *PEPC-Zmetr2* (*PEPC::Zmetr2*), or *Shrunken1-Zmetr2* (*Sh1::Zmetr2*) were germinated and RNA was extracted from specific tissues of one-month old plants for Northern analysis. Transgene expression was examined in roots, white portions of leaves prior to its emergence and chloroplast development as well as the green portion of the same leaves, and the first fully-expanded leaf. Transgene expression was also examined in specific tissues of flowering plants including mature leaves, husk leaves, tassel, pollen, ovary, silk, and kernel.

Expression from *RbcS:: Zmetr2* was high in non- to pale-green young leaves and husk leaf (Fig.5.1.A and D); low expression in silk and ovary (Fig.5.1.E and F); low to no expression in green leaves, and tassel (Fig. 5.1.B, C,

G, and H), whereas expression from *PEPC:: Zmetr2* was high in mature green leaves (Fig.5.1.B and C); low expression in root (Fig.5.1.H); low to no expression in non-green leaf, husk leaf, silk, ovary, and tassel (Fig. 5.1.A, D-G). Tubulin serves as the loading control.

In one-month old plants, expressions from *RbcS::Zmetr2* were higher in young leaves, i.e. leaf 11 to leaf 15, whereas expressions from *PEPC::Zmetr2* were higher in more mature leaves, i.e. leaf 4 to leaf 10 (Fig. 5.2.A). In plants at flowering, expressions from *RbcS::Zmetr2* were low in all leaves, from leaf 10 to leaf 19, whereas expressions from *PEPC::Zmetr2* were higher in young leaves, i.e. leaf 15 to leaf 18 (Fig. 5.2.B). At different developing stages of leaf 12, expressions from *RbcS::Zmetr2* were higher at younger stages, i.e. tip green to 3 weeks after white stage, whereas expressions from *PEPC::Zmetr2* were higher at more mature stages, i.e. 4 weeks after white stage to 4 weeks after flowering (Fig. 5.2.C).

Sh1::Zmetr2 was expressed in every tissue examined, including non- to pale-green young leaves, mature green leaves, kernels, ovaries, and silks (Fig.5.3.A-E). Among seven individual transformants, L45 has the highest level of expression, and L10 has a moderate level of expression (Fig.5.3.A-E). Tubulin serves as the loading control

Chlorophyll contents follow the development of the leaf in mutants expressing Zmetr2 driven by leaf-specific promoters

To investigate the development of photosynthesis machinery in mutants expressing *RbcS::Zmetr2* or *PEPC::Zmetr2*, chlorophyll a/b contents and ratios were examined in *RbcS::Zmetr2* L24 (38-24-37) and *PEPC::Zmetr2* L47 (39-47-1). Chlorophyll was extracted by 90% acetone from leaves at different stages, and chlorophyll a/b contents were calculated base on fresh weight.

In one-month old plants, both *RbcS::Zmetr2* and *PEPC::Zmetr2* had lower chlorophyll a contents in young leaves (i.e. leaf 12 of *RbcS::Zmetr2* and leaf 10 of *PEPC::Zmetr2*), and the content increased as the leaf gets more developed (i.e. leaf 6) (Fig.5.4, top). In plants at flowering, chlorophyll a contents were higher in mature green leaves (i.e. leaf 12 to leaf 16 of *RbcS::Zmetr2* and leaf 10 to leaf 16 of *PEPC::Zmetr2*), and decreased in older leaves (i.e. leaf 17 to leaf 19 of *RbcS::Zmetr2* and leaf 17 to leaf 18 of *PEPC::Zmetr2*) (Fig.5.4, middle). In leaf 12 of both mutants, chlorophyll a contents follow the development of the leaf (Fig.5.4, bottom). The chlorophyll a content started low as the leaf was young, increased as the leaf gets more mature, and went back down as the leaf senesced (Fig.5.4, bottom). The chlorophyll b content showed a similar trend to chlorophyll a content, and the chlorophyll a/b ratio is within a range from 2.72 to 3.87 (data not shown).

Dominant negative Zmetr2 mutant confer ethylene insensitivity

To determine whether expression of the dominant negative mutant receptor Zmetr2 conferred a state of ethylene insensitivity, the expression of the ethylene-inducible ACC oxidase gene *ACO35* was examined. Transformant lines homozygous for *Rubisco-Zmetr2*, *PEPC-Zmetr2*, or *Shrunken1-Zmetr2* were germinated in the dark for nine days, treated with air or 20 ppm ethylene for 25 hr. Total RNA was extracted from both the ethylene and air-treated plants for Northern analysis.

The inductions of *ZmACO35* in mutants expressing RbcS::Zmetr2 and PEPC::Zmetr2 were not conclusive, and need further investigations (Fig.5.5.A). The expression from *ZmACO35* was induced by 20ppm ethylene treatment in the transgenic control plant, whereas no inductions of *ZmACO35* were seen in L10 and L45 expressing Sh1::Zmer2 (Fig.5.5.B). Tubulin serves as the loading control.

To investigate the state of ethylene insensitivity conferred by the expression of the dominant negative mutant receptor *Zmetr2*, NPQ (non-photochemical quenching) was examined in *Sh1::Zmetr2* L45. Both transgenic control plants and *Sh1::Zmetr2* L45 were grown in low light and treated without or with 20ppm ethylene overnight. NPQ was elicited by 146 PFD and the value of initiation NPQ was collected by IMAGING-PAM Chlorophyll Fluorescence System. The value is represented by a scale of colors, from black to white, and

color blue corresponds to a higher value than color green. The ethylene treatment lowered the initiation NPQ in control plants (Fig. 5.6, top), whereas similar values of initiation NPQ were seen in untreated and treated mutants (Fig.5.6, bottom)

DISCUSSION

Maize utilizes the C4 pathway, in which Rubisco is active in bundle sheath cells, and PEP Carboxylase is active in mesophyll cells. *RbcS::Zmetr2* and *PEPC::Zmetr2* showed leaf-specific expressions from *Zmetr2* at different stages. *RbcS* promoter drives the expression from *Zmetr2* at younger stages of the leaf, whereas *PEPC* promoter directs the expression in mature green leaves. Although the expression from *Zmetr2* in particular leaf cell types driven by each promoter has not yet been characterized, the state of ethylene insensitivity could be conferred at different stages in these mutants. By studying these two mutants, *RbcS::Zmetr2* and *PEPC::Zmetr2*, the role of ethylene can be revealed not only in different cell types but also at different stages of leaf development.

However, the state of ethylene insensitivity may not be easily determined by having no induction of ethylene responsive gene, e.g. *ZmACO35*. The tissue-specific promoter may confine the expression from *Zmetr2* in certain cell types, e.g. *RbcS::Zmetr2* expression in bundle sheath cells and *PEPC::Zmetr2* in mesophyll cells. Therefore, in either mutant, the leaf is a mixture of both ethylene

sensitive and insensitive cells, and the whole leaf based analysis only reveals the average of ethylene responses from all cells.

Sh1::Zmetr2 expresses not only in kernel, but also in many other tissues, e.g. leaf and silk. The high expression of *Sh1::Zmetr2* in L45 has conferred a state of ethylene insensitivity on *ZmACO35* induction and initiation NPQ by ethylene treatment. Although tissue-specific expression is not achieved, the function of mutant receptor *Zmetr2* is determined in this mutant. Nevertheless, together with *RbcS::Zmetr2* and *PEPC::Zmetr2* mutants, the role of ethylene in kernel development can still be revealed indirectly.

ACS knockdown mutants of maize produce less ethylene (16) but still express wild-type and functional ethylene receptors. In the Arabidopsis *etr1-1* mutant, the production of endogenous ethylene is not affected, but the mutant receptor fails to bind ethylene (4). Therefore, both *Zmacs* and *etr1-1* mutants are experiencing a lower level of ethylene in the whole plant throughout their lifespan, which may create unintended side effects and difficulties on verifying any specific role of ethylene in plant (34). By using a flower-specific promoter from Petunia (fbp1), the *etr1-1* transformed carnation showed strong insensitivity to ethylene without the unwanted side effects of earlier experiments (35). From approaches in this study, the expression of the dominant negative mutant receptor gene, *Zmetr2*, showed a leaf- or kernel- specificity, which could avoid side effects, e.g.

poor root development, normally seen in constitutive ethylene insensitive transgenic plants (34).

As a model system, many studies have been carried out in Arabidopsis but the roles of ethylene in maize are not as well understood. Because of some known advantages of ethylene insensitivity, ethylene antagonists, e.g. 1-MCP and NBD, are used extensively to control plant growth and development (36-39). However, most of the applications were focused on extending the vase life of cut flowers and the display life of potted plants, regulating fruit ripening, and preventing the deleterious effects of ethylene in vegetables. A few studies in the amelioration of stress responses, e.g. wounding and cold-water in tomato have been done (40, 41). In maize, it is still unknown how a pharmacological approach to manage ethylene signaling can be used to achieve better yield. Because of the high economic value of this cereal, this study was carried out to provide more information concerning the roles of ethylene and how manipulating ethylene signaling can improve yield.

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Figure 5.1. The patterns of expressions from *RbcS::Zmetr2* or *PEPC::Zmetr2* in different maize tissues (A) white portion of emerging leaves from one-month old plants (B) green portion of emerging leaves from one-month old plants (C) mature green leaves from plants at flowering (D) husk leaves (E) silks (F) ovaries (G) tassels (H) roots. C: the transgenic control line; L24: the mutant expressing *RbcS::Zmetr2*; L11: the mutant expressing *PEPC::Zmetr2*. *α-Tubulin* serves as the loading control.

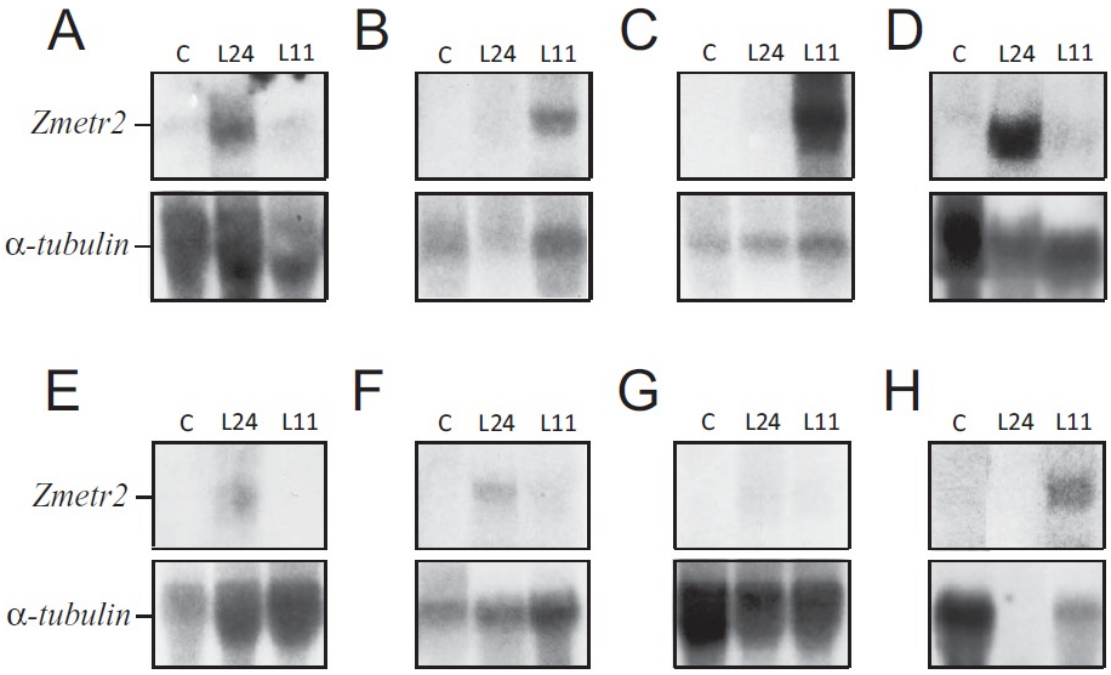

A
B
C
D
E
F
G
H
C
L24
L11
Zmetr2
α-tubulin

Figure 5.2. The patterns of expressions from *RbcS::Zmetr2* or *PEPC::Zmetr2* at different leaf stages (A) Expressions from *RbcS::Zmetr2* in leaf 5 to leaf 15 (left) and from *PEPC::Zmetr2* in leaf 4 to leaf 14 (right) of one-month old plants (B) Expressions from *RbcS::Zmetr2* in leaf 10 to leaf 19 (left) and from *PEPC::Zmetr2* in leaf 10 to leaf 18 (right) of plants at flowering (C) expressions from *RbcS::Zmetr2* (left) and *PEPC::Zmetr2* (right) in leaf 12 at different stages. TG: tip green; 2W-6W: 2-6 weeks after white stage; 2-4F: 2-4 weeks after flowering

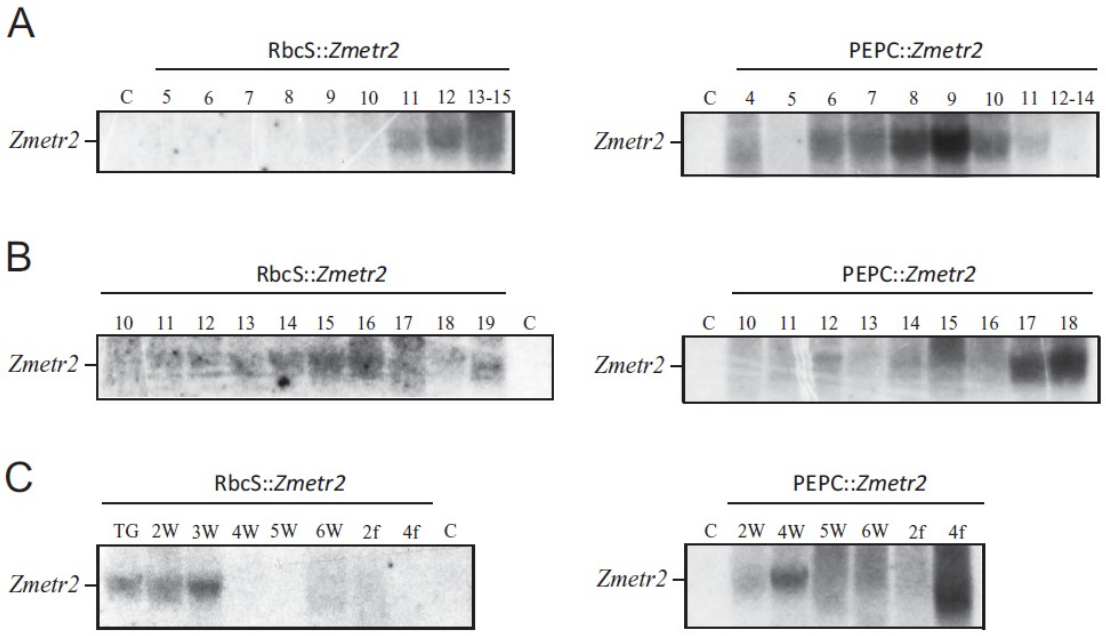

A
RbcS::Zmetr2
C 5 6 7 8 9 10 11 12 13-15
Zmetr2
PEPC::Zmetr2
C 4 5 6 7 8 9 10 11 12-14
Zmetr2
B
RbcS::Zmetr2
10 11 12 13 14 15 16 17 18 19 C
Zmetr2
PEPC::Zmetr2
C 10 11 12 13 14 15 16 17 18
Zmetr2
C
RbcS::Zmetr2
TG 2W 3W 4W 5W 6W 2f 4f C
Zmetr2
PEPC::Zmetr2
C 2W 4W 5W 6W 2f 4f
Zmetr2

Figure 5.3. The pattern of expressions from *Sh1::Zmetr2* in different maize tissues (A) mature green leaves from one-month old plants (B) kernels (C) ovaries (D) silks (E) white portion (left) and green portion (right) of emerging leaves from one-month old plants. C: the transgenic control line; L2, L10, L11, L35, L45, L6, L13: individual transformants expressing *Sh1::Zmetr2*; *α-Tubulin* serves as the loading control.

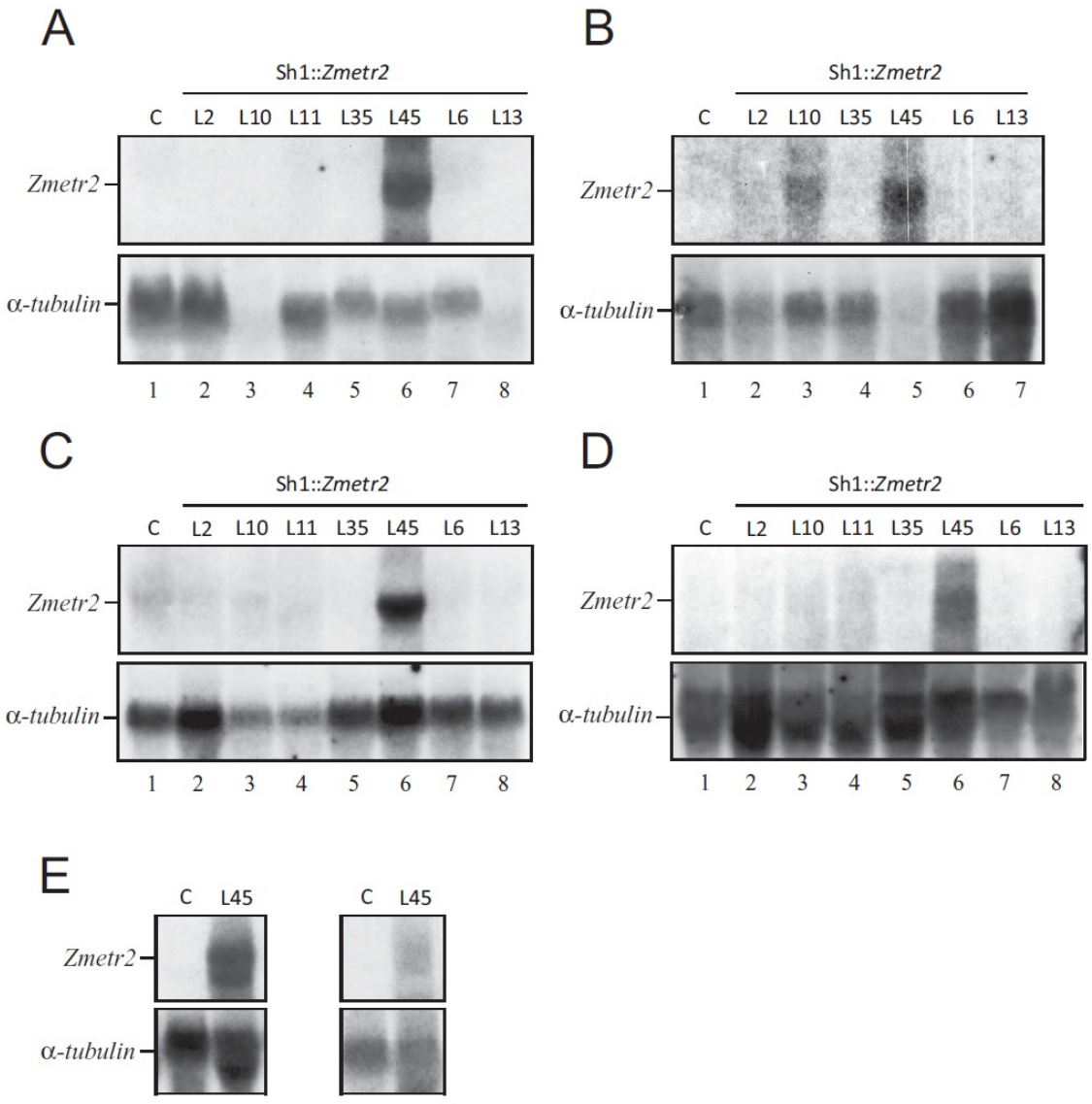
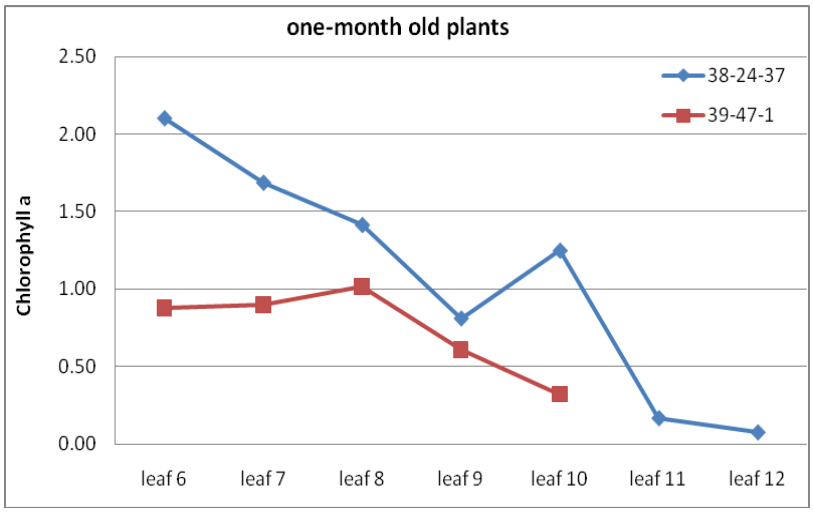
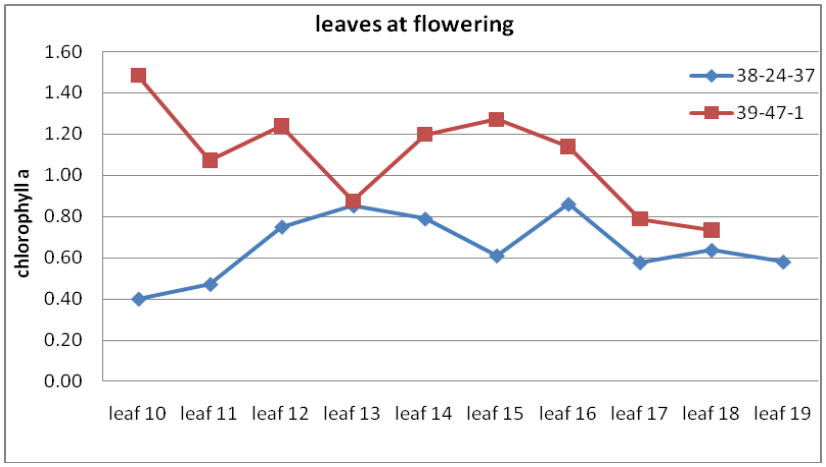

A
Sh1::Zmetr2
C L2 L10 L11 L35 L45 L6 L13
Zmetr2
α-tubulin
1 2 3 4 5 6 7 8
B
Sh1::Zmetr2
C L2 L10 L35 L45 L6 L13
Zmetr2
α-tubulin
1 2 3 4 5 6 7
C
Sh1::Zmetr2
C L2 L10 L11 L35 L45 L6 L13
Zmetr2
α-tubulin
1 2 3 4 5 6 7 8
D
Sh1::Zmetr2
C L2 L10 L11 L35 L45 L6 L13
Zmetr2
α-tubulin
1 2 3 4 5 6 7 8
E
C L45
C L45
Zmetr2
α-tubulin

Figure 5.4. Chlorophyll a contents in mutants expressing *RbcS::Zmetr2* or *PEPC::Zmetr2* at different leaf stages: (Top) Chlorophyll a contents in leaf 6 to leaf 12 of a one-month old plant expressing *RbcS::Zmetr2* (blue) and in leaf 6 to leaf 10 of a one-month old plant expressing *PEPC::Zmetr2* (red); (Middle) Chlorophyll a contents in leaf 10 to leaf 19 of a plant expressing *RbcS::Zmetr2* (blue) and in leaf 10 to leaf 18 of a plant expressing *PEPC::Zmetr2* (red) at flowering; (Bottom) Chlorophyll a contents in leaf 12 of plants expressing *RbcS::Zmetr2* (blue) and *PEPC::Zmetr2* (red) at different stages. TG: tip green; 2W-6W: 2-6 weeks after white stage; 2-4F: 2-4 weeks after flowering


one-month old plants
Chlorophyll a
2.50
2.00
1.50
1.00
0.50
0.00
leaf 6
leaf 7
leaf 8
leaf 9
leaf 10
leaf 11
leaf 12
38-24-37
39-47-1


leaves at flowering
chlorophyll a
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
leaf 10
leaf 11
leaf 12
leaf 13
leaf 14
leaf 15
leaf 16
leaf 17
leaf 18
leaf 19
38-24-37
39-47-1

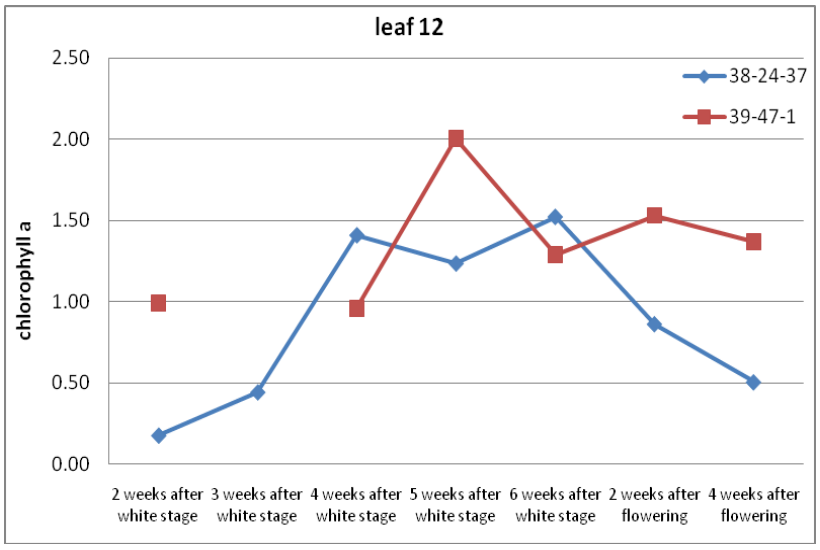

leaf 12
chlorophyll a
2.50
2.00
1.50
1.00
0.50
0.00
2 weeks after white stage
3 weeks after white stage
4 weeks after white stage
5 weeks after white stage
6 weeks after white stage
2 weeks after flowering
4 weeks after flowering
38-24-37
39-47-1

Figure 5.5. Expressions from *ZmACO35* induced by 20ppm ethylene treatment in mutants expressing (A) *RbcS::Zmetr2*, *PEPC::Zmetr2*, or (B) *Sh1::Zmetr2*. C: the transgenic control line; L1, L6, L11, and L24: individual lines expressing *Rbcs::Zmetr2*; L11, L25, and L47: individual lines expressing *PEPC::Zmetr2*; L2, L10, L11, L35, L45, L6, and L13: individual lines expressing *Sh1::Zmetr2*; A: air control; E: ethylene treatment; *α-Tubulin* serves as the loading control.

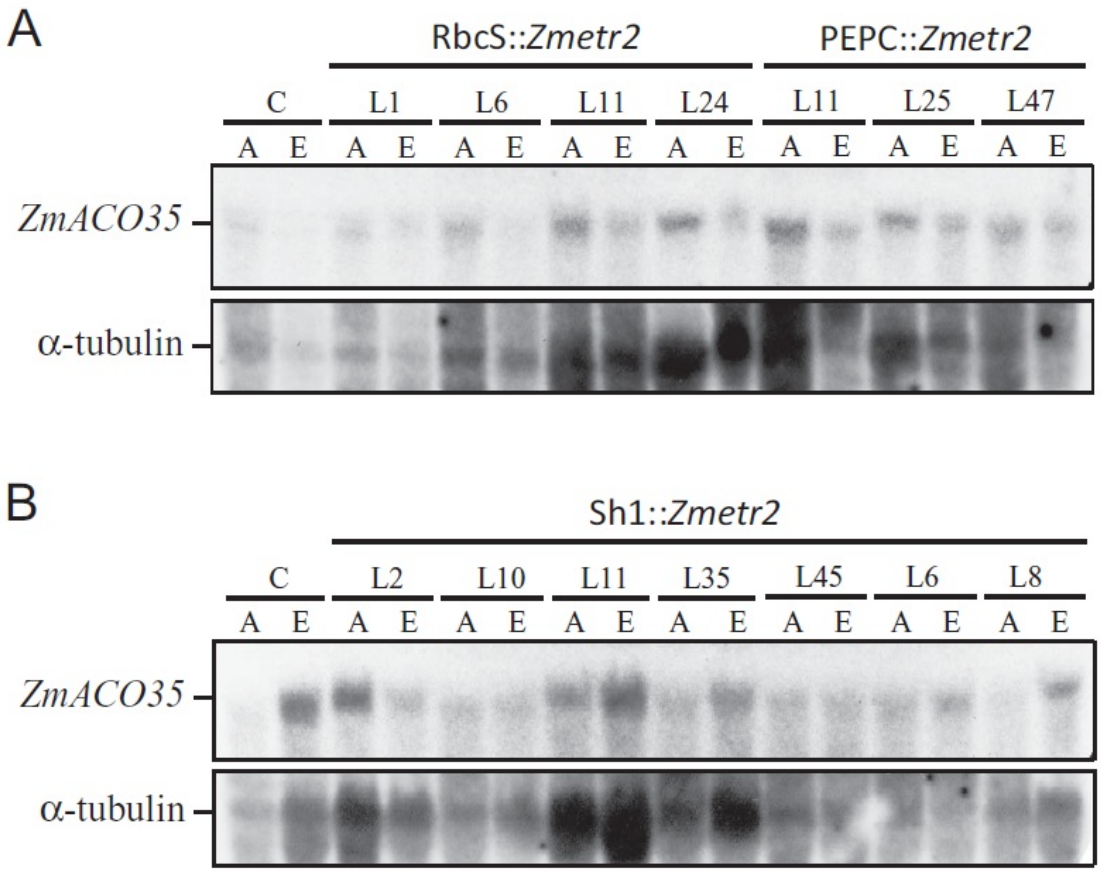
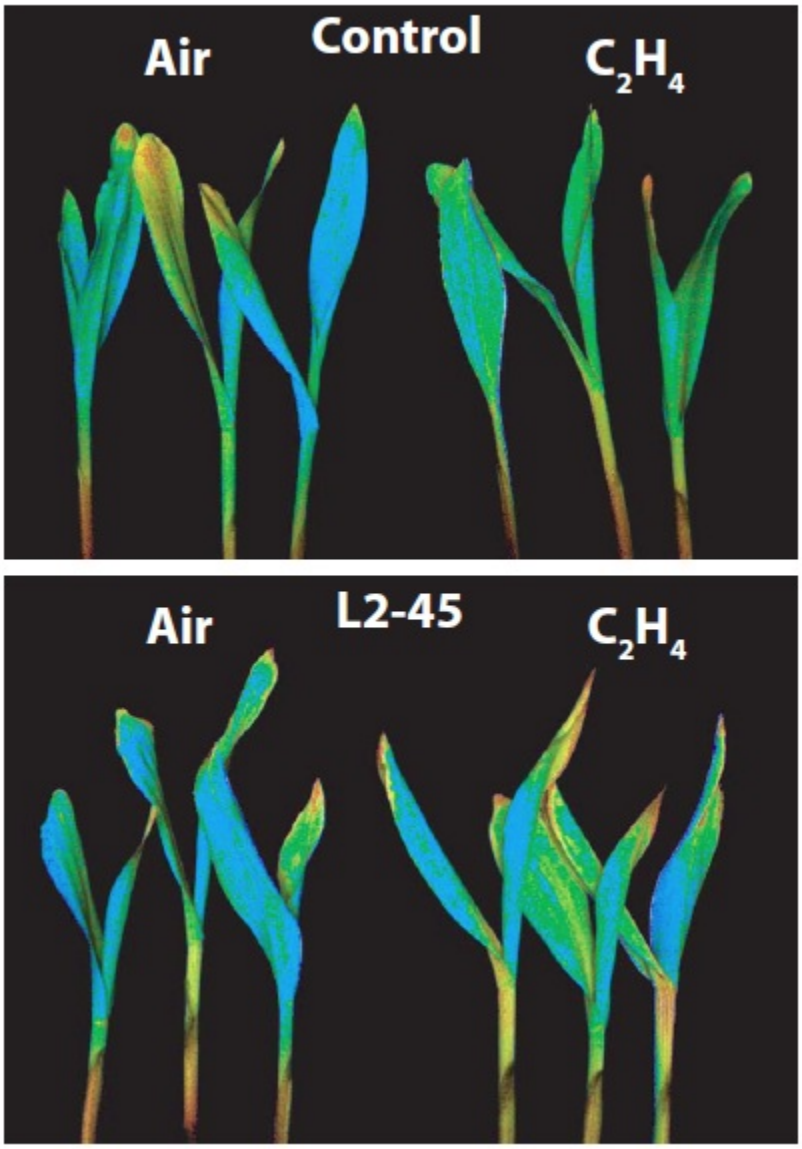

A
RbcS::Zmetr2
PEPC::Zmetr2
C
L1
L6
L11
L24
L11
L25
L47
A E A E A E A E A E A E A E A E
ZmACO35
α-tubulin
B
Sh1::Zmetr2
C
L2
L10
L11
L35
L45
L6
L8
A E A E A E A E A E A E A E A E
ZmACO35
α-tubulin

Figure 5.6. NPQ response to 146 PFD of air and 20ppm ethylene-treated seedlings (Top) NPQ response of air (left) and 20ppm ethylene-treated (right) transgenic control plants (Bottom) NPQ response of air (left) and 20ppm ethylene-treated (right) mutant plants expressing *Sh1::Zmetr2*. Color blue corresponds to a higher NPQ value than color green.

NPQ Response to 146 PFD of Air and 20 ppm Ethylene-Treated Seedlings



CHAPTER 6

CONCLUSIONS

In this study, the function of ethylene in maize was examined from several aspects. ACC synthase (ACS) and ACC oxidase (ACO) are two key enzymes for ethylene biosynthesis. The study of gene expression and regulation of *ZmACS*s and *ZmACO*s in maize roots has provided a better understanding on the role of ethylene in root cap development and cell elongation. Ethylene receptors perceive ethylene and initiate the signaling cascade. The analysis of the functional conservation of ethylene receptors between maize and Arabidopsis has provided insight into the similarity of ethylene signaling on a molecular level despite the difference in sequence and types of receptors in the two species. Although much of the signaling machinery is conserved, aspects of the ethylene response may differ between these species because plant organs or tissues may exhibit differences in sensitivity to this hormone. Tissue-specific ethylene insensitive plants can reveal roles of ethylene in different organs of the plant and the consequences of ethylene insensitivity to the growth and development of a plant.

From real time qRT-PCR analysis, the results show that *ZmACS* and *ZmACO* gene family members are expressed in maize roots and demonstrate distinct patterns of expression for *ZmACS* and *ZmACO* gene family members. From in situ RNA localization analyses, *ZmACS* and *ZmACO* gene family

members show cell specific patterns of expression in maize roots and *ZmACS6* mRNA could be readily detected in the peripheral cells of the root cap. These findings share similarities and differences with what has been observed in deepwater rice and Arabidopsis (1-4). As the product of the reaction catalyzed by ACC oxidase is ethylene, the location of ACO expression is likely the best indicator of where ethylene is actually produced. Expression of *ZmACS* and *ZmACO* in the root proper appears to occur in largely spatially separate cell types where *ZmACS7* expression in the inner cortex is at least one cell layer away from the phloem-specific expression of *ZmACO*. Transport of ACC to and from roots has been reported (5-9) Thus, ACC transport through the phloem might enable ACC synthesized in distal regions of the root to reach the cells of the developing phloem where ACO is expressed

From *Zmacs2* and *Zmacs6* mutants, loss of *ZmACS2* and *ZmACS6* expression results in loss of more than 85% of ethylene production in roots of the *Zmacs2/Zmacs6* double mutant suggests that either *ZmACS7* is responsible for the remaining 15% of ethylene production or that any additional unidentified *ZmACS* gene family members make a relatively minor contribution to the total production of ethylene The study in *Zmacs2-1/6-1* double mutant showed no gross alteration in root development suggesting either that ethylene is not essential for root development or that the residual level of ACC generated in the mutants is sufficient. However, detailed analysis revealed that ethylene controls root cap development and xylem cell elongation. Ethylene has been shown to

regulate cell elongation in roots, typically by repressing cell elongation while promoting radial expansion although exceptions have been observed (10-12). The inhibition of ethylene biosynthesis by aminoethoxyvinylglycine (AVG) resulted in the activation of cells in the QC and calyptrogen of maize roots (13), results that are consistent with the increase in root cap cell number and decrease in root cap cell size observed in *Zmacs* roots which could be reversed by exogenous ACC. In contrast, a role for ethylene in promoting cell division within the QC was observed in Arabidopsis roots (14), which may be due to structural differences between the root types or reflect a difference between the two species.

From the root growth assay in *Zmacs2-1*, *Zmacs6-1*, and *Zmacs2-1/6-1*, *Zmacs6* roots exhibited a greater rate of growth when growth was largely unimpeded. In contrast, growth of *Zmacs6-1* roots in soil was significantly reduced relative to *Zmacs2-1* or wild-type roots, resulting in a significantly reduced root biomass. The increase in expression of *ZmACS6* in soil-grown, wild-type roots correlated with an increase in ethylene evolution that was not seen in *Zmacs6-1* roots. Ethylene signaling has also been shown in tomato to be necessary for root growth in response to physical resistance (15, 16). Therefore, the results suggest that ethylene plays a similar role in maize and that *ZmACS6* serves to regulate root growth in response to soil conditions.

When maize ethylene receptors were analyzed in Arabidopsis, dominant negative *Zmetr2b* and *Zmers1b* mutant receptors are able to confer ethylene insensitivity. In triple response assay, hypocotyl growth in seedlings expressing maize mutant receptor was substantially greater than WT seedlings and similar to the growth of the ethylene insensitive mutant, *ein2-5*. Root growth in lines expressing maize mutant receptor was greater than WT seedlings but slightly reduced relative to *ein2-5* roots. In light-grown seedlings expressing maize mutant receptor, cotyledon expansion and the emergence of the first true leaves were not as inhibited by growth on 20 µM ACC as in WT seedlings and were similar to that observed in *ein2-5* and *etr1-1* seedlings. Root growth in *Zmetr2b*- or *Zmers1*-expressing seedlings grown in the presence of 20 µM ACC was greater than in WT seedlings but less than in *ein2-5* and *etr1-1* seedlings. qPCR analysis demonstrated a lower expression of maize mutant receptor in roots than in leaves. Dose response assay and Northern analysis on ethylene-inducible genes revealed that expression of *Zmetr2b* or *Zmers1b* confers a high level of insensitivity to ethylene. The state of ethylene insensitivity in the hypocotyl conferred by *Zmetr2b* or *Zmers1b* expression is maintained over a wide range of ACC concentrations with a reduction in sensitivity to ethylene in roots. No expression of either *chiB* or *PDF1.2* gene was observed in *Zmetr2b* or *Zmers1b* plants in the presence or absence of ethylene or in *ein2-5* or *etr1-1* plants as would be expected for these ethylene insensitive mutants.

The N-terminal domain of *Zmers1b(1-350)* is sufficient to confer ethylene insensitivity demonstrating that the putative His-kinase domain is not required for *Zmers1b* function. It has been reported that expression of the N-terminal 349 amino acids of *etr1-1*, i.e., *etr1-1(1-349)* can confer ethylene insensitivity in Arabidopsis (17). Interestingly, expression of *Zmetr2b(1-386)* was unable to cause ethylene insensitivity, suggesting that the truncated peptide was not capable of ethylene output signaling. It is possible that the extent of their conservation with endogenous receptors may determine their interaction and therefore their effectiveness, if the function of the N-terminal region of receptor mutants, such as *etr1-1(1-349)* or *Zmers1b(1-350)*, is mediated through interactions with endogenous receptors. ZmERS1 is approximately 73% identical with Arabidopsis ERS1 but ZmETR2 is only 45% identical with Arabidopsis ETR2 (18). It is also possible the C-proximal sequence missing in *Zmetr2b(1-386)* is required for the dominant function exhibited by the full-length *Zmetr2* mutant receptor.

etr1-9;ers1-3 is a double knockout mutant that does not express ETR1 or ERS1 (19). Loss of ETR1 and ERS1 expression results in growth phenotypes more severe than those observed for *ctr1*, which itself is characterized by constitutive ethylene signaling (20). The *Zmetr2b* or *Zmers1b* transgene was introduced into the *etr1-9;ers1-3/+* mutant through crosses with *T:Zmetr2b* line L9 or *T:Zmers1b* line L11. F2 progeny containing *Zmetr2b* or *Zmers1b* included plants that were *etr1-9;ers1-3* and such plants were substantially smaller than

plants exhibiting wild type growth. These results demonstrate that *Zmetr2b* and *Zmers1b* fail to rescue the small growth phenotype of the *etr1-9;ers1-3* double mutant, indicating that the function of *Zmetr2b* and *Zmers1b*, as *etr1-1* (21), receptors is dependent on the expression of subfamily 1 members.

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