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Analysis of the functional conservation of ethylene receptors between maize and Arabidopsis

Jui-Fen Chen · Daniel R. Gallie

Abstract Ethylene, a regulator of plant growth and development, is perceived by specific receptors that act as negative regulators of the ethylene response. Five ethylene receptors, i.e., ETR1, ERS1, EIN4, ETR2, and ERS2, are present in Arabidopsis and dominant negative mutants of each that confer ethylene insensitivity have been reported. In contrast, maize contains just two types of ethylene receptors: ZmERS1, encoded by *ZmERS1a* and *ZmERS1b*, and ZmETR2, encoded by *ZmETR2a* and *ZmETR2b*. In this study, we introduced a Cys to Tyr mutation in the transmembrane domain of ZmERS1b and ZmETR2b that is present in the *etr1-1* dominant negative mutant and expressed each protein in Arabidopsis. Mutant Zmers1b and Zmetr2b receptors conferred ethylene insensitivity and Arabidopsis expressing *Zmers1b* or *Zmetr2b* were larger and exhibited a delay in leaf senescence characteristic of ethylene insensitive Arabidopsis mutants. *Zmers1b* and *Zmetr2b* were dominant and functioned equally well in a hemizygous or homozygous state. Expression of the Zmers1b N-terminal transmembrane domain was sufficient to exert dominance over endogenous Arabidopsis ethylene receptors whereas the Zmetr2b N-terminal domain failed to do so. Neither Zmers1b nor Zmetr2b functioned in the absence of subfamily 1 ethylene receptors, i.e., ETR1 and ERS1. These results suggest that Cys65 in maize ZmERS1b and ZmETR2b plays the same role that it does in 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.

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
ACO	ACC oxidase
β-ME	β-Mercaptoethanol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
MS	Murishige and Skoog
PFD	Photon flux density
SDS	Sodium dodecyl sulfate
SSPE	150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA
TE	Tris–EDTA

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Introduction

The plant hormone ethylene regulates diverse aspects of plant growth and development, including germination; leaf, stem, and root growth; sex determination; fruit ripening; organ abscission; leaf and flower senescence; and cell death of the cereal endosperm (Mattoo and Suttle 1991; Abeles et al. 1992; Dolan 1998; Bleecker and Kende 2000; Schiefelbein 2000; Young and Gallie 2000; Klee 2004; Lin et al. 2009). Ethylene also regulates responses to adverse growth conditions, such as hypoxia, mechanical impedance, and pathogen attack (Feldman 1984; Ecker and Davis

1987; Mattoo and Suttle 1991; Abeles et al. 1992; Morgan and Drew 1997).

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 (Yang and Hoffman 1984). Both ACS and ACO are encoded by multigene families: e.g., the Arabidopsis genome contains nine *ACS* genes that exhibit cell specific and overlapping expression (Liang et al. 1992; Zarembinski and Theologis 1994; Tsuchisaka and Theologis 2004). The *ACO* gene family may be composed of up to 17 members although not all may function as ACC oxidases (Tsuchisaka et al. 2009). 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 (Gallie and Young 2004).

Following its production, ethylene is perceived by binding to endoplasmic reticulum-localized receptors (Chen et al. 2002), of which five different types (i.e., ETR1, ERS1, EIN4, ETR2, and ERS2) are present in Arabidopsis (Bleecker et al. 1998; Chang and Shockey 1999; Chang and Stadler 2001; Wang et al. 2002; Chang and Bleecker 2004; Stepanova and Alonso 2005; Lin et al. 2009). 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 (Kieber et al. 1993; Hua and Meyerowitz 1998; Clark et al. 1998). 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 transcription factors including EIN3/EIL and ERF (Chao et al. 1997; Solano et al. 1998; Alonso et al. 1999).

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 (Schaller 1997; Chang and Stewart 1998; Chang and Stadler 2001; Lohrmann and Harter 2002). 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 (Chang et al. 1993; Hua et al. 1995, 1998; Gamble et al. 1998; Sakai et al. 1998; Moussatche and Klee 2004). 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 (Wang et al. 2003). Despite the distinction between these two subfamilies based on His-kinase activity, mutants of ETR1 lacking His-kinase activity remain competent to rescue the *etr1-7;ers1-2* mutant phenotype in which ETR1 expression is lacking and ERS1 expression is substantially reduced (Wang et al. 2003). 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 (Hall and Bleecker 2003; Wang et al. 2003) and ectopic expression of any subfamily II receptor fails to rescue the *etr1-7;ers1-2* mutant (Wang et al. 2003). 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 (Schaller et al. 1995; O'Malley et al. 2005; Chen et al. 2010).

Mutations of ethylene receptors resulting in constitutive signaling have been described (Bleecker et al. 1988; Chang et al. 1993; Hua et al. 1995, 1998). 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 (Bleecker et al. 1988; Guzmán and Ecker 1990; Chang et al. 1993; Chen and Bleecker 1995). The N-terminal 349 amino acid residues are sufficient to confer ethylene insensitivity (Gamble et al. 2002). Loss of EIN2 expression also disrupts ethylene signaling and results in ethylene insensitivity (Alonso et al. 1999). 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 based on sequence conservation and domain structure: the first type with homology to Arabidopsis ERS1 is referred to as ZmERS1 and the second type that is the likely homolog of Arabidopsis ETR2 is referred to as ZmETR2 (Gallie and Young 2004). 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 (Anderson 1945; Rhoades 1951). 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 (Yau et al. 2004), suggesting that the family structure of ethylene receptors in maize may be a general feature of monocots.

In this report, we have investigated the conservation of maize ethylene receptor function with those in Arabidopsis. A Cys to Tyr mutation was introduced at amino acid 65 in the transmembrane domain of ZmERS1b and ZmETR2b 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. The expression of each mutant maize receptor 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 Zmers1b N-terminal transmembrane domain was expressed. Interestingly, expression of the mutant Zmetr2b 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 mutant maize receptor 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 the dominance of both mutant maize receptors is dependent on subfamily 1 ethylene receptors in Arabidopsis, indicating substantial functional conservation between maize and Arabidopsis ethylene receptors.

Materials and methods

Plasmid constructs and mutagenesis

The cDNAs of *ZmETR2b* and *ZmERS1b* were obtained by RT–PCR from B73 maize RNA and cloned into pGEM-T Easy (Promega, Madison WI, USA). To generate the *Zmetr2b* and *Zmers1b* 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 ZmETR2b: 5′-CATCGTGCT CT<u>A</u>CGGCCTCACGC-3′ and ZmERS1b: 5′-GTTTATA GTTCTCT<u>A</u>TGGGGCAAC-3′, in which the mutation (i.e.,

G to A) is underlined. The region encoding Zmetr2b (1-386) or Zmers1b(1-350) were obtained by PCR from the full length *Zmetr2b* and *Zmers1b* constructs, respectively. 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 *Zmetr2b* or *Zmers1b* 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 0.25× MS agar plates with or without ACC or AgNO₃ 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 were 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 1 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 0.25× 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 H₂O, and resolved on a 1.2% agarose-formaldehyde gel. PCR-generated *Zmetr2b* and *Zmers1b* 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. Where indicated, the membrane was reprobed for *eEF1A* 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× PCR buffer, 0.4 μ 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: 95°C/5 min (1 cycle); 95°C/30 s, 55°C/30 s, 72°C/1 min (35 cycles); and a final extension at 72°C/5 min (1 cycle). To detect the presence of *Zmetr2b*, a forward primer, *ZmETR2*-F1, 5′-ATGGTGGTGGGAACGGC GCCGTGCGGGG-3′, and a reverse primer, *ZmETR2*-R1, 5′-TGCAGTCTGGAAGGAATTCCGAGCTTCC-3′ were used. To detect the presence of *Zmers1b*, a forward primer, *ZmERS1*-F1, 5′-ATGGACGGATGTGATTGCATCGA-3′, and a reverse primer, *ZmERS1*-R1, 5′-AACAGCTAGAAA ATCATTGCGAGCACG-3′ were used. To detect the presence of *AtETR1*, a forward primer, *AtETR1*-F1, 5′-GCGG TTGTTAAGAAATTACCCATCACACT-3′, and a reverse primer, *AtETR1*-R1, 5′-ATCCAAATGTTACCCTCCATC AGATTCAC-3′ were used. To detect the presence of the T-DNA insertion in the *etr1-9* mutant, a forward primer, *AtETR1*-F1, and a reverse primer, T_DNA-L, 5′-CATTT TATAATAACGCTGCGGACATCTAC-3′ were used. To detect the presence of wild-type *AtERS1*, a forward primer, *AtERS1*-F1, 5′-CAGAGAGTTCTGTCACTCCTGGAAAT GGT-3′, and a reverse primer, *AtERS1*-R1, 5′-CACAACC GCGCAAGAGACTTTAGCAATAGT-3′ were used. To detect the presence of the T-DNA insertion in the *ers1-3* mutant, a forward primer, *AtERS1*-F2, 5′-GAACAGGGAA TTGTTTCTCAAGAAGAAAGC-3′, and the reverse primer, T_DNA-L were used.

qPCR analysis

Plant material was frozen in liquid nitrogen, ground to a fine powder, and 100 mg was resuspended in 1 ml TRI-ZOL® Reagent (Invitrogen, Carlsbad CA, USA). Following centrifugation, the supernatant was extracted with 200 μl chloroform and centrifuged to separate the phases. RNA was precipitated from the aqueous phase using isopropyl alcohol, the RNA pellet washed with 75% ethanol and resuspended in RNase-free H_2O. 1 μg RNA was used to obtain the first-strand cDNA by Omniscript RT Kit (Qiagen, Valencia CA, USA) in a 20 μl reaction. The qPCR analysis was performed using a iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules CA, USA) in 25 μl reactions containing 1× SYBR Green SuperMix 500 nM forward and reverse primers and 10 ng cDNA. Reactions were carried out using the following conditions: 95°C/5 min (1 cycle); 95°C/30 s, 55°C/30 s, 72°C/30 s (35 cycles). To detect the presence of *Zmetr2*, a forward primer, ZmETR2-F5, 5′-GAGTTCAAACGGTCAGATAA GTCATGG-3′, and a reverse primer, ZmETR2-R8, 5′-TCG GCTCAAGTCTGAAGACGCC-3′ were used. To detect the presence of *Zmers1*, a forward primer, ZmERS1-F3, 5′-GGCTGCAGTCCGTGTACCTCTTC-3′, and a reverse primer, ZmERS1-R4, 5′-AACAGCTAGAAAATCATTG CGAGCACG were used. Protein phosphatase PP2A (At1g13320) was used as the reference gene for the quantitation of *Zmetr2* and *Zmers1* expression in Arabidopsis leaves and roots. To detect the expression of PP2A, a forward primer, PP2A-FW, 5′-AGTATCGCTTCTCGCTC CAG-3′ and a reverse primer, PP2A-RV, 5′-GTTCTCCA CAACCGCTTGGT-3′ were used. The efficiency of PCR was determined by five 10-fold serial dilutions of the template DNAs in triplicate.

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 0.25× 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 *T:Zmetr2b* line L9 or line *T:Zmers1b* line L11. Screening for the presence of the *ers1-3* allele in F1 progeny from each cross was performed by PCR, and plants that were *T:Zmetr2b*;*etr1-9/+*;*ers1-3/+* or *T:Zmers1b*;*etr1-9/+*;*ers1-3/+* were allowed to self pollinate. F2 seeds were germinated

on 0.25× MS medium and plants exhibiting the small growth phenotype characteristic of *etr1-9;ers1-3* double null 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 Zmetr2b and Zmers1b mutant receptors 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 (Gallie and Young 2004). 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 (Gallie and Young 2004). 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. 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. 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. 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 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. 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. 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. 1(C)). Little to no *Zmers1b* expression was observed in line L12 (lane 7, middle panel, Fig. 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. 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 (Neljubow 1901). Wild-type (WT) seedlings grown in the dark on 20 μM ACC exhibited these characteristics (top panel, Fig. 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. 1(D); Table 1). Root growth in lines expressing *Zmetr2b* was greater than WT seedlings but slightly reduced relative to *ein2-5* roots (Table 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 (Rodríguez et al. 1999). 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. 1(D); Table 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. 1(D); Table 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 (Smalle
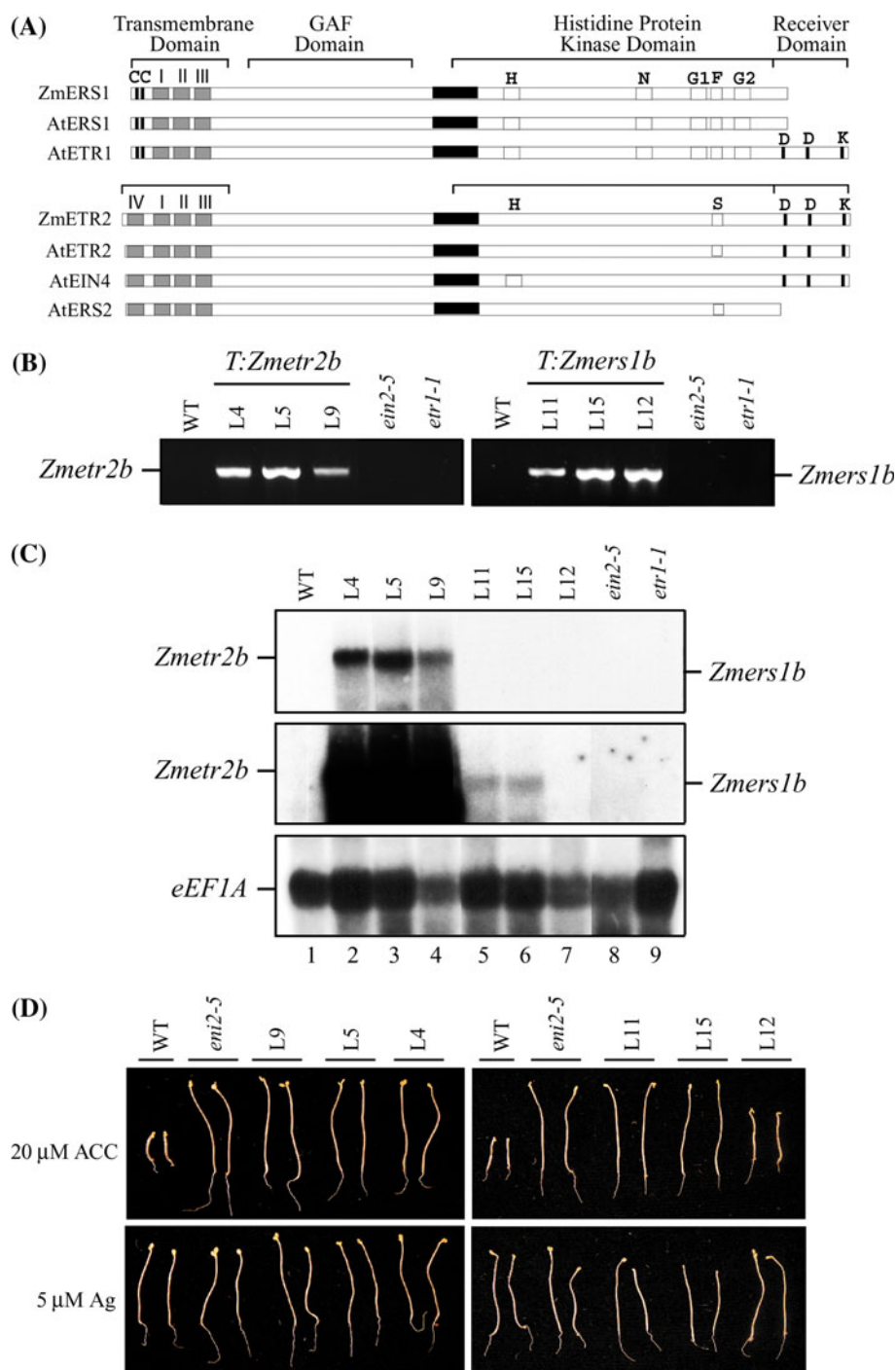
et al. 1997). Light-grown WT seedlings exhibited these characteristics in the presence of 20 μM ACC whereas growth was normal in the absence of ACC (Fig. 2(A)). In Zmetr2b-expressing seedlings, cotyledon expansion and the emergence of the first true leaves were not as inhibited by growth on 20 μM ACC as in WT seedlings and was similar to that observed in ein2-5 and etr1-1 seedlings (Fig. 2(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, T:Zmetr2b seedlings (line L9) demonstrated that Zmetr2b expression in roots was 1.7% of the level in leaves (Fig. 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 was also greater than that of WT seedlings in the presence of 20 μM ACC (Fig. 2(A)). Root growth was also greater than in WT seedlings but less

◀ **Fig. 1** Zmetr2b and Zmers1b function as dominant negative mutants in Arabidopsis. (**A**) Comparison of ZmERS1 with Arabidopsis subfamily I receptors, i.e., AtETR1 and AtERS1, and comparison of ZmETR2 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 (Hua et al. 1998) 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-coil region is indicated by the *black box*. (**B**) PCR amplification of the *Zmetr2b* and *Zmers1b* transgenes from three lines containing *Zmetr2b* (i.e., L4, L5, L9) and *three lines* containing *Zmers1b* (i.e., L11, L15, L12) 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 *Zmetr2b* or *Zmers1b* lines germinated in the dark for 10 days. The level of *Zmetr2b* and *Zmers1b* expression was measured using a mixture of *Zmetr2b* and *Zmers1b* probes after a 24 h (*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 an 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 1

than in *ein2-5* seedlings and was greater than in *Zmetr2b*-expressing seedlings (Fig. 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(B)), consistent with the greater level of root growth observed in *Zmers1b* seedlings relative to *Zmetr2b* seedlings (Fig. 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* (Guzmán and Ecker 1990). 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). 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) but an increase in leaf size (Fig. 3(A)) and a delay in leaf senescence (Fig. 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(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).

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. 4; Table 3). A slight reduction in root growth in hemizygous *Zmetr2b* seedlings relative to roots of

Table 1 Expression of mutant Zmetr2b and Zmers1b 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	$P = 0.371$
T:Zmetr2b L4	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	$P = 0.122$
T:Zmetr2b L5	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	$P < 0.0001$
T:Zmetr2b L9	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	$P = 0.349$
T:Zmers1b L11	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	$P = 0.086$
T:Zmers1b L15	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	$P = 0.921$
T:Zmers1b L12	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

homozygous *Zmetr2b* seedlings was observed (Table 3). These results suggest that expression of *Zmetr2b* and *Zmers1b* can exert dominance when present in a hemizygous state.

(A)

(B)
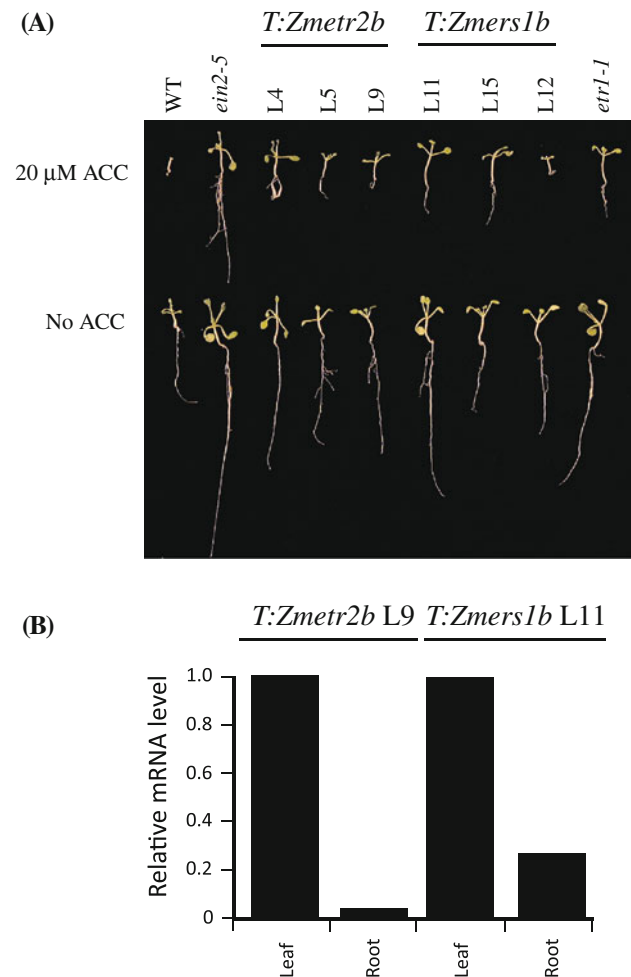
Fig. 2 Expression of *Zmetr2b* and *Zmers1b* confers ethylene insensitivity in light-grown Arabidopsis seedlings. In (**A**), the same lines used in Fig. 1 were germinated in the light for 10 days on media in the presence or absence of 20 μM ACC. One representative seedling is shown for each line. In (**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

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. 5), which was confirmed by quantitative measurements (Fig. 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. 5) and full inhibition was achieved by approximately 2.5 μM ACC (Fig. 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. 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. 5). A slight reduction in hypocotyl growth was observed in *T:Zmers1b* line L11 seedlings at higher ACC concentrations (Fig. 6) with a few seedlings exhibiting an apical hook (Fig. 5). *T:Zmetr2b* line L9 roots were slightly shorter than WT roots when grown in the absence of ACC

Table 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

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

Fig. 3 Arabidopsis expressing *Zmetr2b* or *Zmers1b* exhibit a larger leaf size and a delay in senescence. The same lines used in Fig. 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
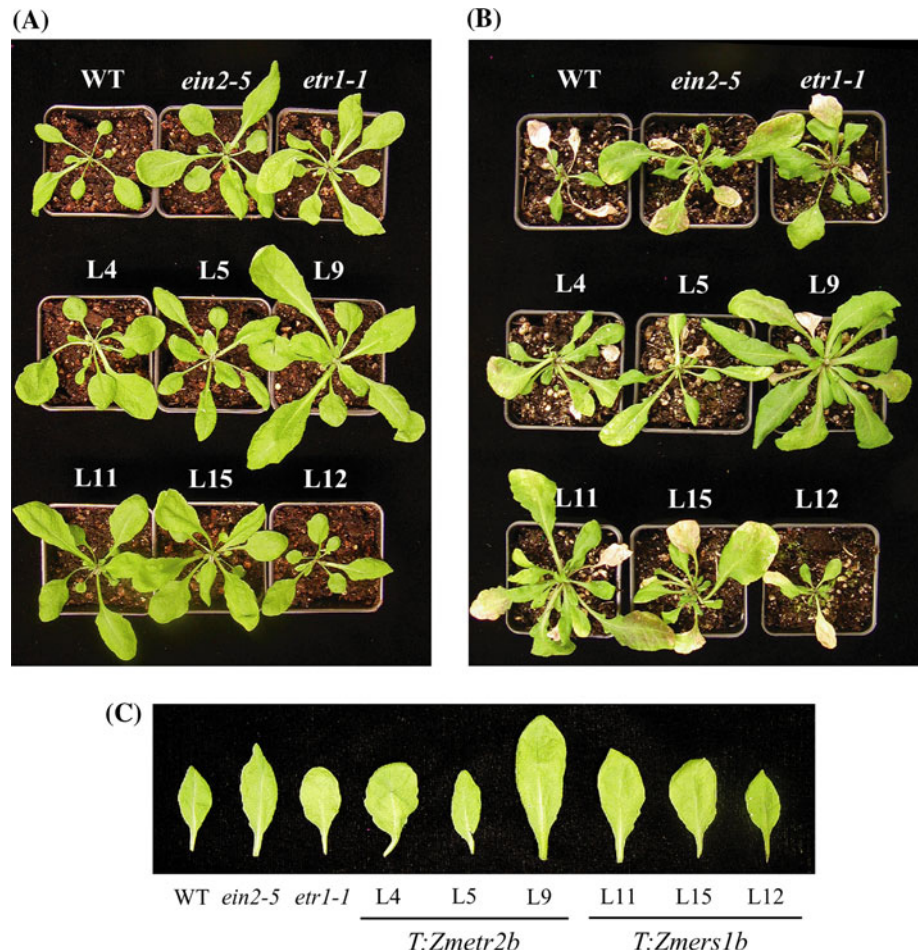
but their growth was not inhibited to same extent as in WT seedlings by increasing concentrations of ACC (Fig. 6). In contrast, *T:Zmers1b* line L11 root growth was inhibited to a similar extent as WT roots in the presence of increasing concentrations of ACC (Fig. 6). These results demonstrate that the 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.
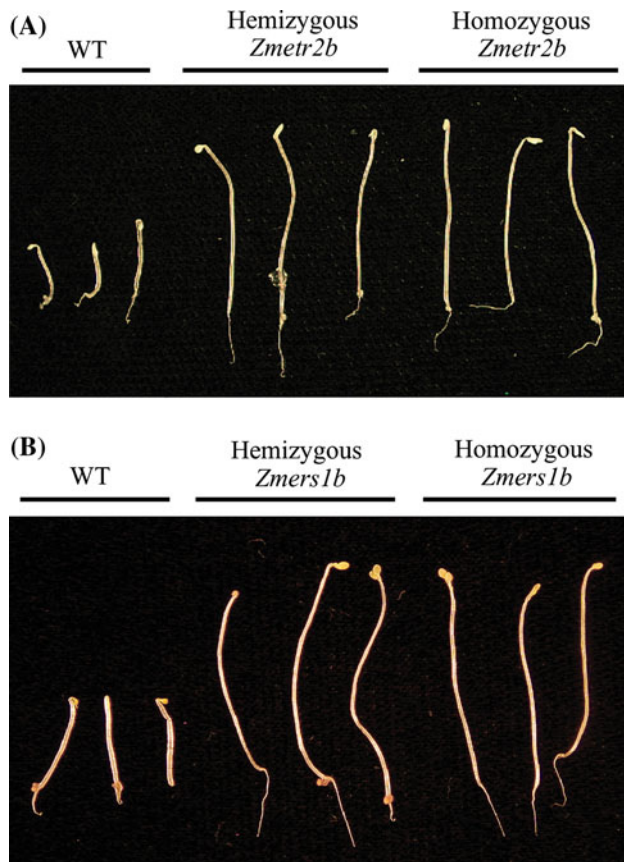
Expression of *Zmetr2b* and *Zmers1b* in Arabidopsis conferred insensitivity to ethylene as measured by the lack of ethylene-mediated hypocotyl growth inhibition. To examine whether the dominance of *Zmetr2b* and *Zmers1b* mutant expression also inhibited ethylene responses at the molecular level, the 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 h 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. 7). As observed in Fig. 1, the *Zmetr2b* or *Zmers1b* probes did not cross react with Arabidopsis ethylene receptor mRNA as demonstrated in WT plants (Fig. 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. 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. 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 (Gamble et al. 2002). 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)

(A)
WT   Hemizygous   Homozygous
            Zmetr2b   *Zmetr2b*

(B)
WT   Hemizygous   Homozygous
            Zmers1b   *Zmers1b*

Fig. 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. Wild-type (*WT*) plants were included as an ethylene sensitive control

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. 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. 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. 8(B)) and in which *Zmers1b(1-350)* expression was easily detected (Fig. 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 (Xie et al. 2006). 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 (Qu et al. 2007). Loss of ETR1 and ERS1 expression results in growth phenotypes more severe than those observed for *ctr1*, which itself is characterized by

Table 3 *Zmetr2b* and *Zmers1b* are dominant when present in a hemizygous state in Arabidopsis
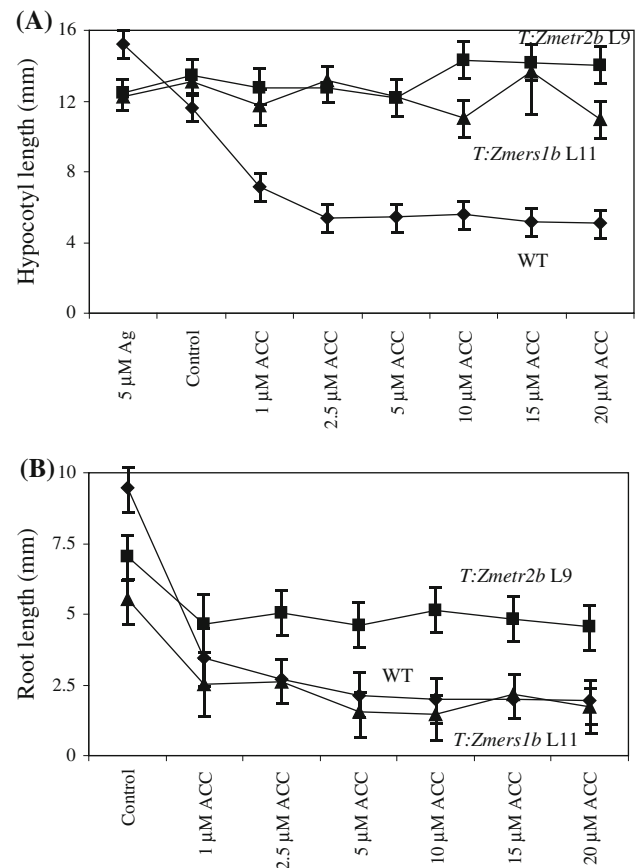
	Hypocotyl length (mm)[a]	*t*-Test	Root length (mm)[a]	*t*-Test
WT	6.37 ± 1.00		3.20 ± 1.14	
Hemizygous *Zmetr2b*	14.0 ± 2.04	$P < 0.001$	6.01 ± 1.64	$P < 0.001$
Homozygous *Zmetr2b*	17.2 ± 1.58	$P < 0.001$	5.83 ± 1.18	$P < 0.001$
WT	8.52 ± 0.65		2.78 ± 0.83	
Hemizygous *Zmerslb*	16.5 ± 4.98	$P < 0.001$	4.78 ± 1.60	$P < 0.001$
Homozygous *Zmerslb*	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
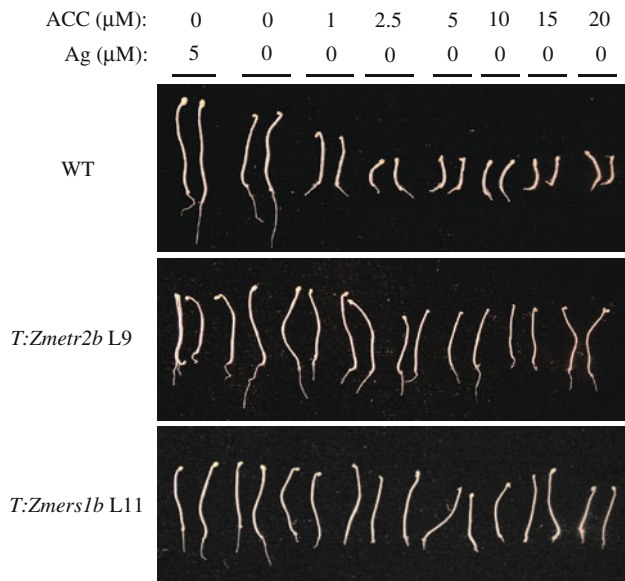
Fig. 5 *Zmetr2b* and *Zmers1b* expression confers ethylene insensitivity over a range of ACC concentrations. Seeds of *T:Zmetr2b* L9 and *T:Zmers1b* L11 were germinated in the presence of either 5 μM AgNO₃ or ACC at the concentrations indicated and grown in the dark for 5 days. Wild-type (*WT*) plants were included as an ethylene sensitive control. Two representative seedlings are shown for each line

Fig. 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 Fig. 5

constitutive ethylene signaling (Kieber et al. 1993). The *etr1-9*;*ers1-3* double mutant plant is extremely small and typically dies before flowering (Qu et al. 2007), 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 (Qu et al. 2007). 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 5). F2 progeny containing *Zmetr2b* included plants that were *etr1-9*;*ers1-3* (Table 5) and such plants were substantially smaller than plants exhibiting wild type growth (Fig. 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 5). Such plants were substantially smaller than plants exhibiting wild type growth (Fig. 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.
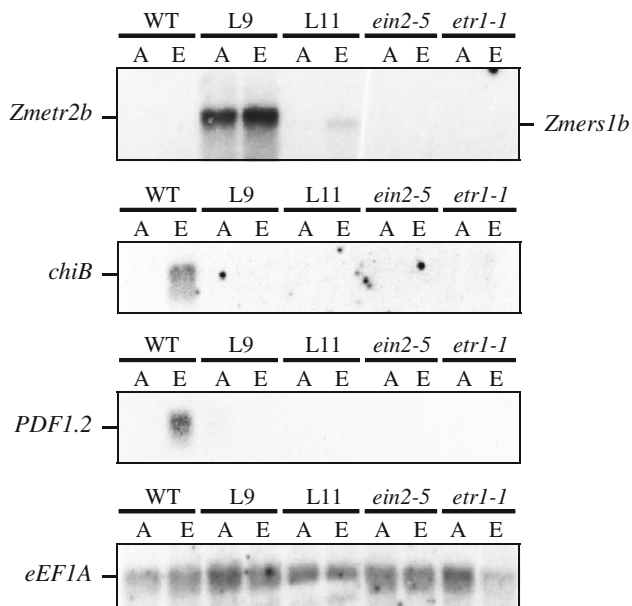
Fig. 7 Induction of ethylene regulated gene expression in Arabidopsis expressing *Zmetr2b* or *Zmers1b*. Northern analysis was performed on total RNA from light-grown *T:Zmetr2b* line L9 and *T:Zmers1b* line L11 plants that were treated either with 100 ppm ethylene (*E*) or air (*A*) for 24 h. Following 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

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, we show 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 (Yau et al. 2004), 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 (Hall et al. 1999; Rodríguez et al. 1999). 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 (Gamble et al. 2002; Xie et al. 2006). 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 (Gamble et al. 2002), 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
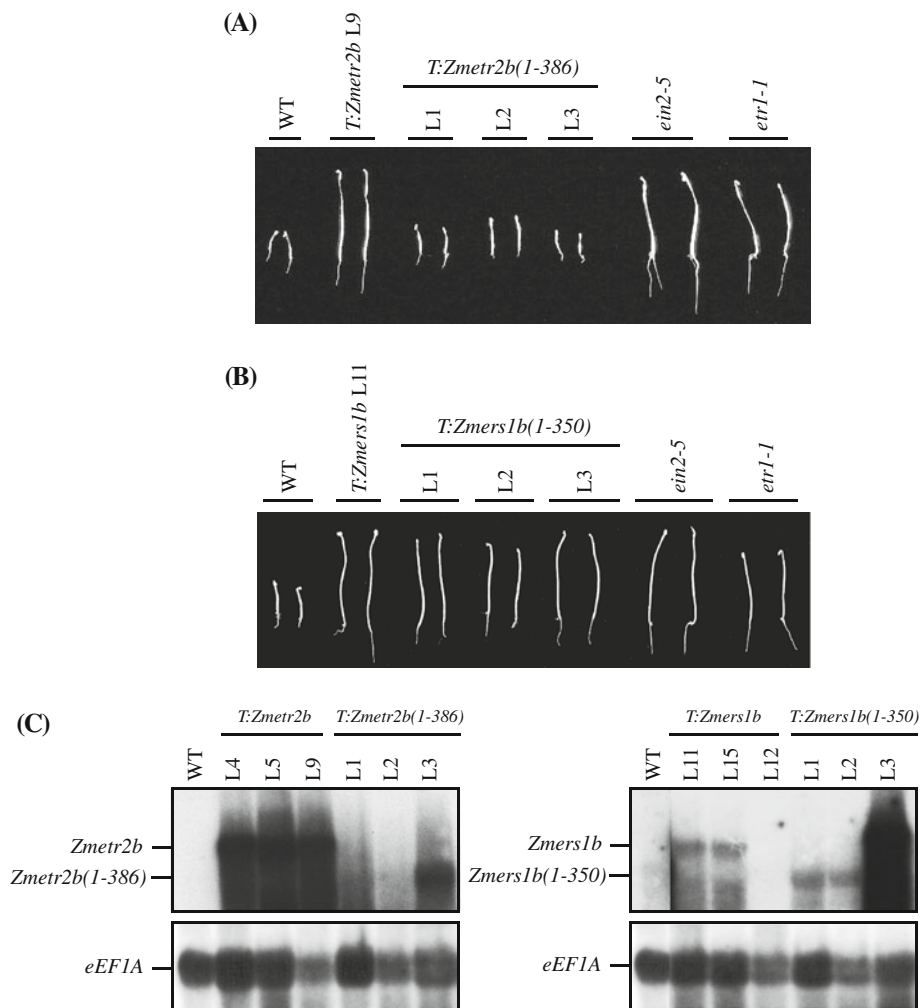
Fig. 8 The N-terminal domain of *Zmers1b* but not *Zmetr2b* is sufficient to exert dominance in Arabidopsis. Arabidopsis was transformed with the portion of the *Zmetr2b* coding region representing the N-terminal domain, i.e., Zmetr2b(1-386), to generate lines *T:Zmetr2b(1-386)* L1, L2, and L3 or transformed 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 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

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 (Xie et al. 2006). 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,

Table 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-l	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 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 × etr1-9;ers1-3/+ cross		Number of small F2 progeny with or without Zmers1b from a T:Zmers1b L11 × 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
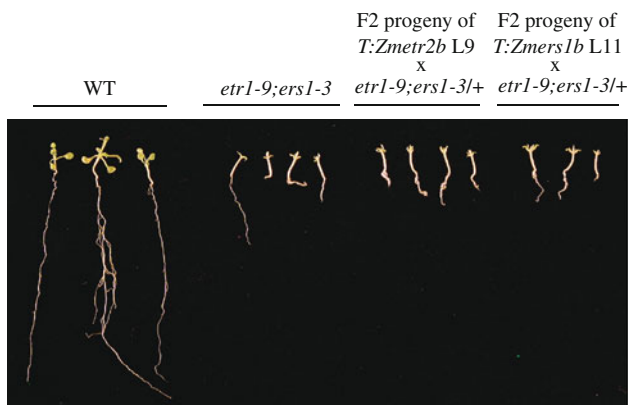
Fig. 9 Zmers1b and Zmetr2b require expression of subfamily I receptors for their function. T:Zmetr2b line L9 or T:Zmers1b line L11 was crossed with etr1-9;ers1-3/+ plants and F1 progeny that were T:Zmetr2b;etr1-9/+;ers1-3/+ or T:Zmers1b;etr1-9/+;ers1-3/+ were selfed. F2 progeny were germinated in the 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 5. Also shown are etr1-9;ers1-3 and WT plants

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 (Gallie and Young 2004). 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) (Xie et al. 2006), 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 (Xie et al. 2006).

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 (Schaller et al. 1995; Gamble et al. 2002; Qu and Schaller 2004; Chen et al. 2010). etr1-1(1-349) covalently dimerizes with ETR1 (Gamble et al. 2002), 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 (Xie et al. 2006), 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 (Gallie and Young 2004). 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 (Xie et al. 2006), 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 (Gao et al. 2008; Chen et al. 2010). 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 (Aravind and Ponting 1997; Ho et al. 2000; Martinez et al. 2002). The GAF domain may function similarly in ethylene receptors as the GAF domain is sufficient to mediate the interaction between Arabidopsis ETR1 and ETR2 (Gao et al. 2008). 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. 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 (Gallie and Young 2004). 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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