

# MAOHUZI6/ETHYLENE INSENSITIVE3-LIKE1 and ETHYLENE INSENSITIVE3-LIKE2 Regulate Ethylene Response of Roots and Coleoptiles and Negatively Affect Salt Tolerance in Rice<sup>1</sup>[OPEN]

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Ethylene plays important roles in plant growth, development, and stress responses. The ethylene signaling pathway has been studied extensively, mainly in *Arabidopsis* (*Arabidopsis thaliana*). However, the molecular mechanism of ethylene signaling is largely unknown in rice (*Oryza sativa*). Previously, we have isolated a set of rice ethylene-response mutants. Here, we characterized the mutant *maohuzi6* (*mhz6*). Through map-based cloning, we found that *MHZ6* encodes ETHYLENE INSENSITIVE3-LIKE1 (*OsEIL1*), a rice homolog of ETHYLENE INSENSITIVE3 (*EIN3*), which is the master transcriptional regulator of ethylene signaling in *Arabidopsis*. Disruption of *MHZ6/OsEIL1* caused ethylene insensitivity mainly in roots, whereas silencing of the closely related *OsEIL2* led to ethylene insensitivity mainly in coleoptiles of etiolated seedlings. This organ-specific functional divergence is different from the functional features of *EIN3* and *EIL1*, both of which mediate the incomplete ethylene responses of *Arabidopsis* etiolated seedlings. In *Arabidopsis*, *EIN3* and *EIL1* play positive roles in plant salt tolerance. In rice, however, lack of *MHZ6/OsEIL1* or *OsEIL2* functions improves salt tolerance, whereas the overexpressing lines exhibit salt hypersensitivity at the seedling stage, indicating that *MHZ6/OsEIL1* and *OsEIL2* negatively regulate salt tolerance in rice. Furthermore, this negative regulation by *MHZ6/OsEIL1* and *OsEIL2* in salt tolerance is likely attributable in part to the direct regulation of *HIGH-AFFINITY K<sup>+</sup> TRANSPORTER2;1* expression and Na<sup>+</sup> uptake in roots. Additionally, *MHZ6/OsEIL1* overexpression promotes grain size and thousand-grain weight. Together, our study provides insights for the functional diversification of *MHZ6/OsEIL1* and *OsEIL2* in ethylene response and finds a novel mode of ethylene-regulated salt stress response that could be helpful for engineering salt-tolerant crops.

The simple hydrocarbon phytohormone ethylene regulates a variety of basic plant processes throughout whole-plant growth and development (Abeles et al., 1992). In *Arabidopsis* (*Arabidopsis thaliana*), genetic studies of ethylene-responsive mutants have uncovered

a linear ethylene signaling framework from hormone perception in the endoplasmic reticulum to transcriptional regulation in the nucleus. Ethylene is sensed by five endoplasmic reticulum-localized receptors that are negative regulators of the signaling pathway (Chang et al., 1993; Hua et al., 1995, 1998; Hua and Meyerowitz, 1998; Sakai et al., 1998). Without ethylene, the receptors may recruit and activate CONSTITUTIVE TRIPLE RESPONSE1 (*CTR1*), which further phosphorylates the C-terminal domain of ETHYLENE INSENSITIVE2 (*EIN2*) to repress the downstream ethylene response (Kieber et al., 1993; Alonso et al., 1999; Ju et al., 2012). When ethylene is present, the receptors and *CTR1* are inactive. The C terminus of *EIN2* is cleaved and translocated to the nucleus to participate in the stabilization and accumulation of *EIN3/ETHYLENE INSENSITIVE3-LIKE1* (*EIL1*) and, consequently, induces ethylene-responsive genes (Chao et al., 1997; Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). *EIN2* and *EIN3/EIL1* are regulated by proteasomal degradation through ETHYLENE INSENSITIVE2 TARGETING PROTEIN1 (*ETP1*) and *ETP2* and ETHYLENE INSENSITIVE3-BINDING F-BOX PROTEIN1 (*EBF1*) and *EBF2*, respectively (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Qiao et al., 2009).

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Semiaquatic rice (*Oryza sativa*) is one of the most important crops in the world. Ethylene plays essential roles in the adaptive responses to the hypoxia conditions of rice (Fukao and Bailey-Serres, 2008; Rzewuski and Sauter, 2008; Ma et al., 2010; Yang et al., 2015). Different from the triple response observed in etiolated Arabidopsis seedlings, ethylene inhibits root growth but promotes coleoptile growth of dark-grown rice seedlings, which is called the double response (Ma et al., 2010, 2013, 2014; Yang et al., 2015). Several homologous genes of Arabidopsis ethylene signaling components have been identified and characterized in rice, including the ethylene receptor genes *ETHYLENE RESPONSE SENSOR1* (*OsERS1*) and *ETHYLENE RESPONSE2* (*OsETR2*), *REVERSION TO ETHYLENE SENSITIVITY1* (*RTE1*) homolog (*RTH1*), *OsCTRs*, *OsEIN2*, and *OsEIL1* (Jun et al., 2004; Mao et al., 2006; Wuriyangan et al., 2009; Zhang et al., 2012; Ma et al., 2013, 2014; Wang et al., 2013). Overexpression of *OsETR2* reduces ethylene sensitivity and delays the floral transition in transgenic rice. Conversely, *OsETR2* RNA interference (RNAi) plants and knockdown mutants show enhanced ethylene sensitivity, early flowering, and increased thousand-seed weight (Wuriyangan et al., 2009). Overexpression of *OsRTH1*, the Arabidopsis *RTH1* in rice, rescues the ethylene-insensitive phenotype of the *RTE1* loss-of-function mutant *rte1-2* (Zhang et al., 2012). Ectopic expression of *OsCTR2* largely complements Arabidopsis *ctr1-1*, while *OsCTR2* loss-of-function plants show several aspects of the constitutive ethylene-response phenotypes (Wang et al., 2013). Based on the double response of rice, our laboratory has isolated a set of rice ethylene-response mutants, and *MAOHUZI7* (*MHZ7*)/*OsEIN2* was identified. The *mhz7* mutant shows complete insensitivity to ethylene in both root and coleoptile growth, whereas *MHZ7-OX* rice seedlings show constitutive or enhanced ethylene response without or with ethylene treatment, respectively (Ma et al., 2013). Recently, the study of *MHZ4* revealed a novel mode of interplay between ethylene and abscisic acid (ABA) in the control of rice growth and development (Ma et al., 2014). *MHZ4* encoded a chloroplast-localized membrane protein homologous to Arabidopsis *ABA4*. *MHZ4* mutation leads to reduced ethylene response in roots but enhanced ethylene response in coleoptiles of etiolated seedlings. And overexpression of *MHZ4* resulted in enhanced and reduced ethylene response in roots and coleoptiles, respectively (Ma et al., 2014). More recently, through analysis of the *mhz5* mutant, we found that the *MHZ5*/carotenoid isomerase-mediated ABA pathway acts downstream of ethylene signaling, and ethylene induces the expression of *MHZ5* and *MHZ4* and drives the metabolic flux of carotenogenesis into ABA biosynthesis to regulate root growth in rice (Yin et al., 2015).

Ethylene is known to play important roles in regulating biotic and abiotic stress responses (Morgan and Drew, 1997; van Loon et al., 2006). Over the years, the functions of components of ethylene biosynthesis and signaling in response to salt stress were investigated in dicotyledonous plants. Our previous studies have

demonstrated that subfamily II ethylene receptor genes from tobacco (*Nicotiana tabacum*), tobacco *HISTIDINE KINASE1* (*NTHK1*) and *NTHK2*, are induced by different stresses (Zhang et al., 2001a, 2001b). *NTHK1* overexpression plants show a salt-sensitive phenotype; however, the presence of 1-aminocyclopropane-1-carboxylic acid suppresses the salt sensitivity (Cao et al., 2006, 2007). The kinase domain and kinase activity of *NTHK1* are functionally differential in the regulation of the salt stress response and growth response (Zhou et al., 2006; Chen et al., 2009). Recently, two *NTHK1*-interacting proteins, including the ankyrin-domain protein *NTHK1 ETHYLENE RECEPTOR-INTERACTING PROTEIN2* (*NEIP2*) and the translationally controlled tumor protein *TRANSLATIONALLY CONTROLLED TUMOR PROTEIN* (*TCTP*), have been identified. *NEIP2* can associate with *NTHK1* to promote growth and stress tolerance (Cao et al., 2015). *TCTP* can stabilize *NTHK1* to reduce the ethylene response but enhance plant growth through the promotion of cell proliferation (Tao et al., 2015). The Arabidopsis *ein2-1* and *ein2-5* mutants also show a salt-sensitive response to salt stress, whereas overexpression of the C-terminal end of *EIN2* in *ein2-5* exhibits a salt-tolerant phenotype (Cao et al., 2007; Lei et al., 2011). The *ctr1-1* and *ebf1-1ebf2-1* mutants that cause *EIN3* accumulation exhibit increased salt tolerance, whereas the *ein3-1* mutant exhibits reduced salt tolerance (Achard et al., 2006; Lei et al., 2011). Recently, Peng et al. (2014) reported that plants pretreated with ethylene show increased tolerance to salt stress, and *EIN3/EIL1* are both necessary and sufficient for salt tolerance. The mutation of *ETHYLENE OVERPRODUCER1* (*ETO1*) function causing enhanced ethylene production promotes soil salinity tolerance by reducing root-to-shoot  $\text{Na}^+$  delivery (Jiang et al., 2013). These studies support that enhanced ethylene production or signaling improves salt tolerance in dicotyledonous plants. However, the roles of ethylene and its signaling components in salt stress responses remain unclear in rice.

Recently, we isolated a set of rice ethylene-response mutants and identified *MHZ7/OsEIN2*, *MHZ4/ABA4*, and *MHZ5* through map-based cloning (Ma et al., 2013, 2014; Yin et al., 2015). In this study, we characterize *mhz6*, a mutant exhibiting ethylene insensitivity in root growth. Through map-based cloning, we found that *mhz6* is a loss-of-function mutation of the previously described *OsEIL1* locus, whose encoded protein is homologous to *EIN3*, the key transcription factor of the ethylene signaling pathway in Arabidopsis (Chao et al., 1997; Mao et al., 2006). We further demonstrate that, different from the incomplete ethylene insensitivity of *ein3* and *eil1* in Arabidopsis, *MHZ6/OsEIL1* and *OsEIL2* differentially regulate ethylene responses of the roots and coleoptiles of etiolated seedlings. We next show that *MHZ6/OsEIL1* and *OsEIL2* negatively regulate salt tolerance in rice, which is different from the positive salt-tolerant regulation of *EIN3/EIL1* in Arabidopsis. Furthermore, this negative regulation by *MHZ6/OsEIL1* and *OsEIL2* in salt tolerance is likely in part attributable to the direct

regulation of *HIGH-AFFINITY K<sup>+</sup> TRANSPORTER2;1* (*OsHKT2;1*) expression and Na<sup>+</sup> uptake in roots. Our results suggest that *MHZ6/OsEIL1* and *OsEIL2*, in contrast to Arabidopsis *EIN3/EIL1*, may play distinct roles not only in ethylene signal transduction but also in regulating salt-stress responses.

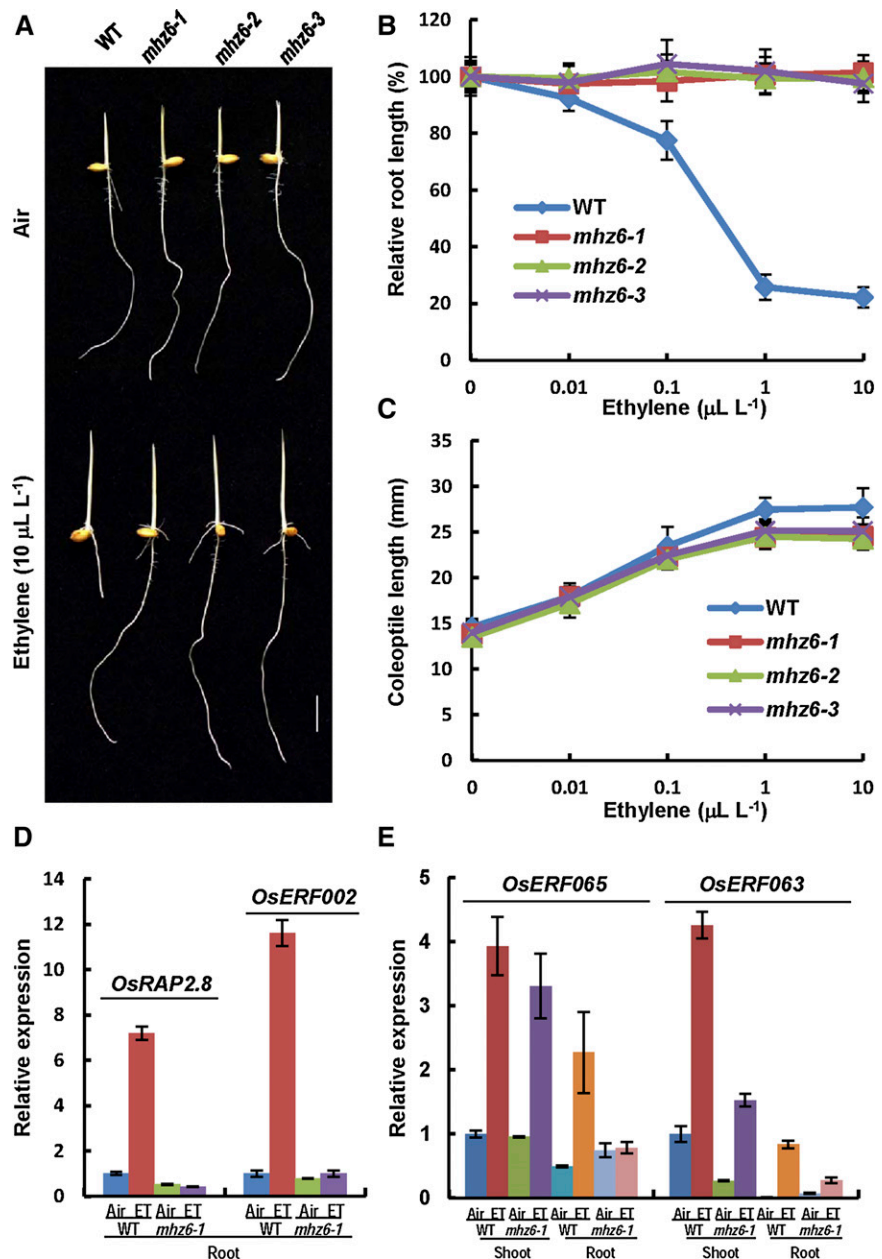
**RESULTS**

**The *mhz6* Mutants Display Insensitivity to Ethylene in Roots**

In an effort to clarify the ethylene signal pathway in a model monocot plant, a set of rice ethylene-response

mutants were isolated in our laboratory (Ma et al., 2013). One group of the mutants, named *mhz6*, was analyzed. Compared with the wild type, etiolated seedlings of three allelic mutants, *mhz6-1*, *mhz6-2*, and *mhz6-3*, had similar phenotypes in air but showed longer roots in 10 μL L<sup>-1</sup> ethylene (Fig. 1A). The ethylene dose-response curve revealed that wild-type root length was drastically reduced under ethylene treatments from 0.1 to 10 μL L<sup>-1</sup>, whereas the mutant root growth was not inhibited by ethylene, indicating a completely ethylene-insensitive phenotype (Fig. 1B). Coleoptile growth of wild-type and *mhz6* mutant seedlings was promoted similarly after ethylene treatment, although the mutants seemed to have slightly shorter coleoptiles (Fig. 1C). These results

**Figure 1.** Characterization of ethylene responses of *mhz6* seedlings. A, Ethylene-insensitive phenotypes of the wild type (WT) and different *mhz6* alleles. Bar = 10 mm. B, Ethylene dose-response curves for relative root length of the wild type and *mhz6* mutants. Values are means ± SD (*n* ≥ 30). C, Ethylene dose-response curves of coleoptile length of the wild type and *mhz6* mutants. Values are means ± SD (*n* ≥ 30). D, Real-time PCR analysis of the expression of ethylene-inducible genes in roots of the wild type and *mhz6-1*. Two-day-old etiolated seedlings were treated with or without 10 μL L<sup>-1</sup> ethylene (ET) for 8 h, and the RNA was isolated for analysis. Values are means ± SD (*n* = 3). E, Real-time PCR analysis of the expression of ethylene-inducible genes in both shoots and roots of the wild type and *mhz6-1*. Details are as in D.



indicate that *mhz6* is completely insensitive to ethylene in root growth and slightly insensitive in coleoptile promotion.

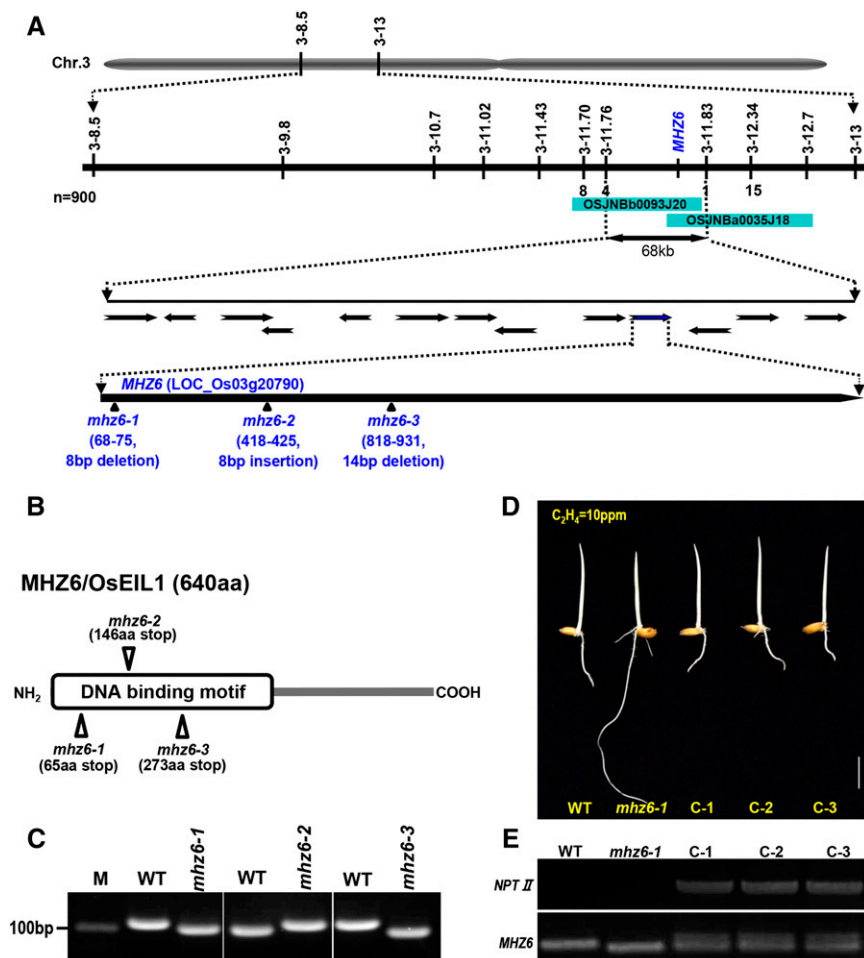
To further examine the ethylene response of the *mhz6* mutants, we determined the expression of ethylene-responsive genes (Ma et al., 2013). *OsRAP2.8* and rice *ETHYLENE RESPONSE FACTOR002* (*OsERF002*) were found to be induced to higher levels by ethylene only in roots of wild-type seedlings but not in roots of *mhz6-1* seedlings (Fig. 1D). *OsERF063* and *OsERF065* were found to be induced by ethylene in both roots and shoots of wild-type seedlings. In *mhz6-1*, the expression of the two genes in roots remained at a very low level after ethylene treatment, while in shoots the ethylene induction of the two genes was similar or mildly reduced when compared with those in the wild type (Fig. 1E). These results indicate that the *mhz6-1* mutant is ethylene insensitive in roots for some ethylene-responsive gene expression.

### Molecular Cloning and Functional Verification

Segregation analysis revealed that the *mhz6-1* ethylene-insensitive phenotype was recessive (Ma et al., 2010). The

*mhz6-1* mutant was crossed with four *indica* cultivars, and 900 segregated mutant individuals from the F2 population were used for fine mapping. Finally, we restricted *MHZ6* to a 68-kb interval between insertion/deletion markers 3-11.76 and 3-11.83 on chromosome 3 (Fig. 2A). Sequencing of all the genes in this region revealed that 8-bp deletions were identified in the exon of Os03g20790 (Fig. 2A). The Os03g20790 locus encoded a homologous protein of EIN3, which is an important transcription factor of ethylene signaling in Arabidopsis (Chao et al., 1997; Fig. 2B). In rice, this gene has been named as *OsEIL1* and analyzed through an ectopic overexpression approach (Mao et al., 2006); however, the ethylene response controlled by *OsEIL1* in plants is still unclear. The *mhz6-2* mutant harbored 8-bp insertions at position 418; *mhz6-3* harbored 14-bp deletions at position 818. All the mutations produced stop codons (Fig. 2, A and B). The mutation was also confirmed by nested PCR through examination of DNA fragment length polymorphism (Fig. 2C).

*MHZ6/OsEIL1* complementation transgenic lines were generated by introducing the pMHZ6C construct into *mhz6-1* plants. The wild-type phenotype was segregated from T1 lines after 10  $\mu\text{L L}^{-1}$  ethylene treatment



**Figure 2.** Molecular characterization of *MHZ6*. **A**, Map-based cloning of *MHZ6*. The *MHZ6* locus was mapped in a 68-kb interval on chromosome 3 between markers 3-11.76 and 3-11.83. The number under the marker indicates the number of recombinant individuals. Mutation sites of three allelic mutants are indicated in the schematic diagram of *MHZ6*. **B**, Mutation sites of the three allelic mutants shown on the schematic structure of the *MHZ6* protein predicted using the SMART software (<http://smart.embl-heidelberg.de>). **C**, Confirmation of mutation sites in *mhz6-1*, *mhz6-2*, and *mhz6-3* by nested PCR analysis. **D**, Functional complementation of the *mhz6-1* mutant. The wild type (WT), *mhz6-1*, and three complementary lines were treated with 10  $\mu\text{L L}^{-1}$  ethylene. Bar = 10 mm. **E**, PCR-based analyses of *NPTII* and *MHZ6* genes in the wild type, *mhz6-1*, and the three complementary lines.

(Fig. 2D), indicating that *MHZ6* complemented the *mhz6-1* phenotype. PCR-based analyses of *NEOMYCIN PHOSPHOTRANSFERASE II (NPTII)* and *MHZ6* with genomic DNA confirmed the transgenic lines (Fig. 2E). All these results indicate that *MHZ6* corresponds to the locus Os03g20790.

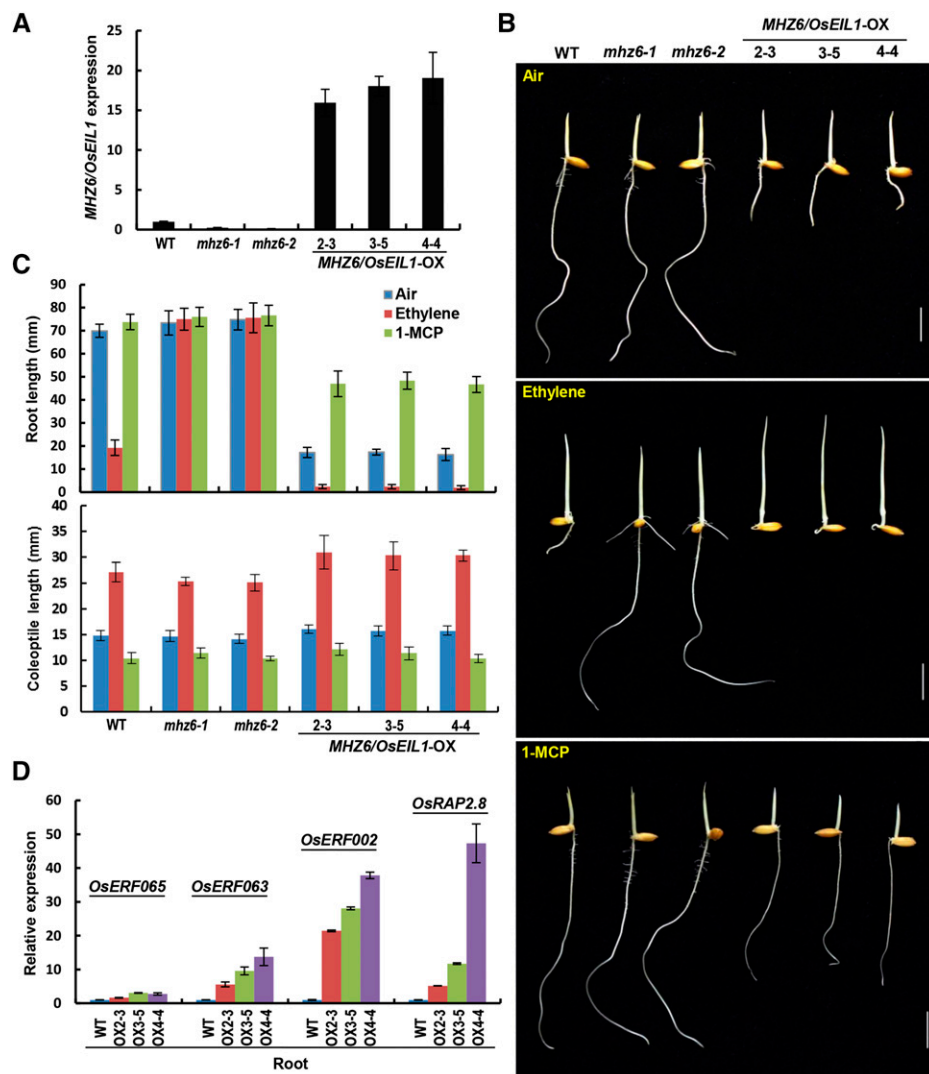
**Overexpression of *MHZ6/OsEIL1* Leads to a Constitutive Ethylene Response**

To further investigate the function of *MHZ6/OsEIL1*, we introduced the plasmid p35S:*MHZ6* containing the *MHZ6/OsEIL1* gene into wild-type plants. *MHZ6/OsEIL1* expression levels of all plant materials were examined by real-time PCR, and three overexpressing (OX) lines (OX-2-3, OX-3-5, and OX-4-4) were chosen for further analysis (Fig. 3A). In air, etiolated seedlings of the three lines all showed shorter roots and slightly longer coleoptiles compared with the wild type, suggesting the presence of a constitutive ethylene response

mainly in roots (Fig. 3, B and C). After ethylene treatment, the overexpression lines showed much shorter and extremely twisted roots, along with slightly longer coleoptiles, in comparison with the wild type and *mhz6* mutants, indicating enhanced ethylene-response phenotypes mainly in roots (Fig. 3, B and C). We further used 1-methylcyclopropene (1-MCP; Serek et al., 1995), a chemical that can tightly bind to the ethylene receptors in plants to block ethylene perception, and examined its effect on the ethylene response of the overexpression lines. The constitutive ethylene response of etiolated seedlings of *MHZ6/OsEIL1*-OX lines is largely eliminated in air-grown roots and completely blocked in coleoptiles by the application of 1-MCP (Fig. 3, B and C).

We further identified *MHZ6/OsEIL1*-regulated ethylene-responsive genes in roots of *MHZ6/OsEIL1*-OX lines. The expression levels of *OsRAP2.8*, *OsERF002*, *OsERF063*, and *OsERF065* were substantially enhanced in comparison with the wild type in air-grown etiolated seedlings,

**Figure 3.** *MHZ6/OsEIL1* overexpression conferred constitutive and enhanced ethylene responses strongly in roots but slightly in coleoptiles in the absence and presence of ethylene, respectively. A, *MHZ6/OsEIL1* expression in etiolated seedlings of the wild type (WT), *mhz6* alleles, and *MHZ6/OsEIL1*-OX transgenic lines by real-time PCR. Values are means  $\pm$  SD ( $n = 3$ ). B, Ethylene-response phenotypes of the wild type, *mhz6* mutants, and *MHZ6/OsEIL1*-OX lines. The etiolated seedlings were grown in air,  $10 \mu\text{L L}^{-1}$  ethylene, or  $1 \mu\text{L L}^{-1}$  1-MCP for 3 d. Bars = 10 mm. C, Length of roots and coleoptiles in the wild type, *mhz6* mutants, and *MHZ6/OsEIL1*-OX lines in response to ethylene and 1-MCP. Values are means  $\pm$  SD ( $n \geq 30$ ). D, Expression of ethylene-inducible genes in roots of the wild type and three *MHZ6/OsEIL1*-OX lines. Values are means  $\pm$  SD ( $n = 3$ ).





which proved the constitutive ethylene response in roots of *MHZ6*-OX lines (Fig. 3D). All these data indicate that *MHZ6* overexpression leads to a constitutive ethylene response and confers an enhanced response to ethylene treatment mainly in roots.

#### Transactivation Analysis and Subcellular Localization of OsEILs

There are six EIL homologs, designated as OsEIL1 to OsEIL6, in rice. Phylogenetic analysis indicates that *MHZ6*/OsEIL1 and OsEIL2 showed the highest similarity to the Arabidopsis EIN3 (Supplemental Fig. S1A). The transcriptional activation abilities of OsEILs were investigated using a dual-luciferase reporter assay system in the Arabidopsis protoplast. *OsEIL* genes were fused to the GAL4 (yeast [*Saccharomyces cerevisiae*] transcription activator Gal4) DNA-binding domain (DBD) coding sequence and constructed into pRT107 to generate effector plasmid pRTBD-OsEILs. Compared with the GAL4-DBD negative control, *MHZ6*/OsEIL1, OsEIL2, OsEIL3, and OsEIL4 strongly activated the reporter gene activity, while OsEIL5 and OsEIL6 showed less activity than the negative control (Supplemental Fig. S1B). These results indicate that OsEIL1 to OsEIL4 can activate transcription, whereas OsEIL5 and OsEIL6 appear to lack this activity. We next determined the subcellular localization of OsEIL1 to OsEIL4. Each gene was fused to the *GFP* gene in a transient expression vector, and the fusion genes driven by the 35S promoter were transfected into Arabidopsis protoplasts. All the OsEILs were located in the nucleus, and the *GFP* control was mainly located in the cytoplasm (Supplemental Fig. S1C). These results indicate that the OsEIL1 to OsEIL4 proteins are nuclear proteins.

#### *OsEIL2* Is Required for Ethylene-Promoted Coleoptile Elongation

The ethylene-response phenotypes of *mhz6* mutants indicated that *MHZ6*/OsEIL1 is fully required for ethylene-inhibited root growth but only slightly required the ethylene-promoted coleoptile elongation. We then examined whether other *OsEILs* have any roles in the regulation of the ethylene response in coleoptiles. To elucidate the function of other *OsEILs*, we generated RNAi constructs of *OsEIL2* and *OsEIL3* and identified an *OsEIL4* knockout mutant (*oseil4*) with Tos17 insertion from the stock seeds in Rice Genome Resource Center (<http://tos.nias.affrc.go.jp/~miyao/pub/tos17/index.html.en>). We also generated overexpression constructs of *OsEIL2*, *OsEIL3*, and *OsEIL4*. All the plasmids were introduced into wild-type rice for further analysis.

For *OsEIL2* transgenic plants, *OsEIL2* expression levels of all plant materials were examined by semi-quantitative PCR, and three knockdown lines (19, 39,

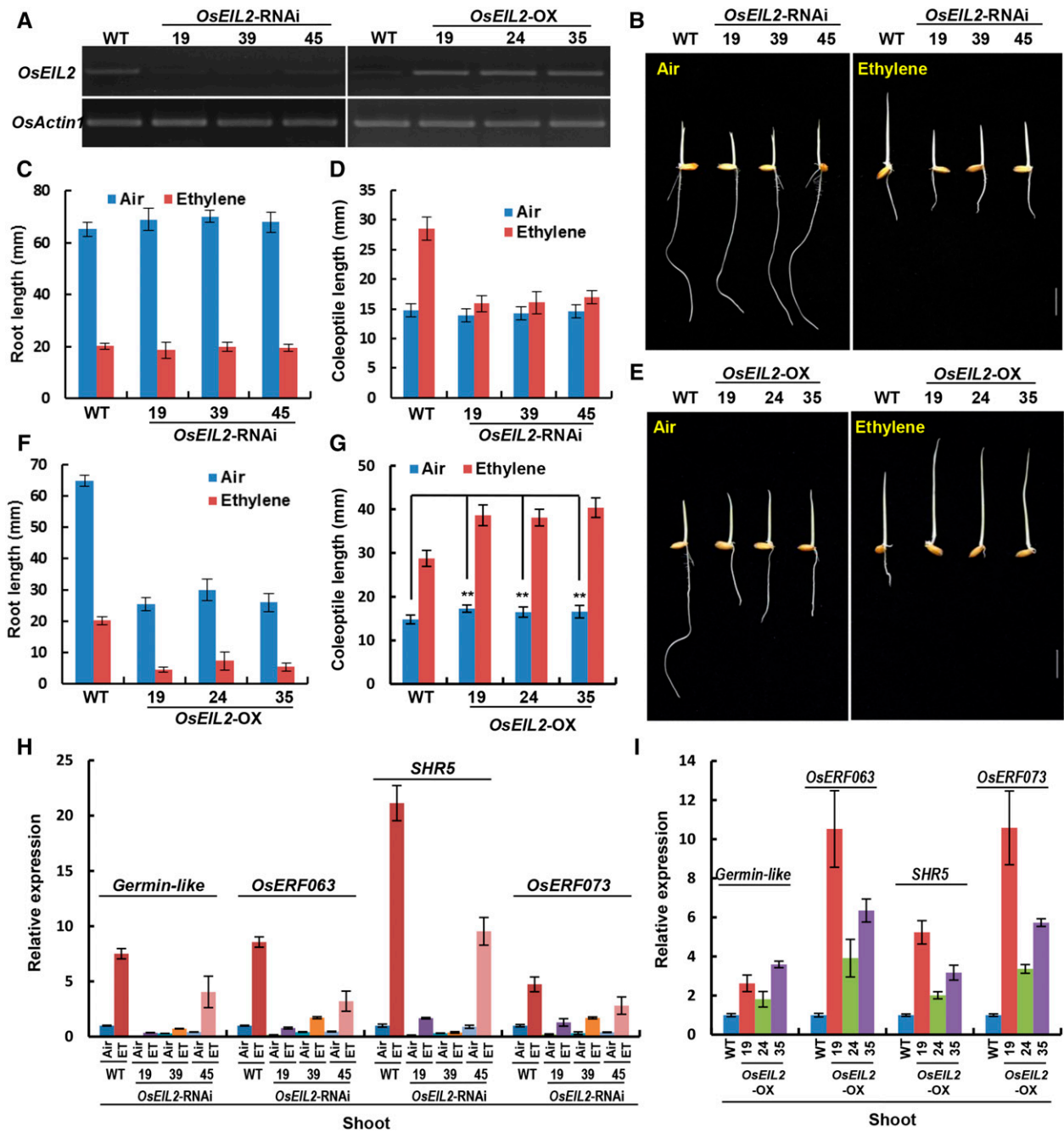
and 45) and three OX lines (19, 24, and 35) were chosen for further analysis (Fig. 4A). Compared with the wild type, the etiolated seedlings of three *OsEIL2*-RNAi lines had almost normal ethylene-response phenotypes in roots but exhibited shorter coleoptiles under  $10 \mu\text{L L}^{-1}$  ethylene (Fig. 4, B–D). These results indicated that knockdown of *OsEIL2* conferred strong insensitivity in ethylene-promoted coleoptile elongation.

We further analyzed the ethylene response of *OsEIL2*-OX lines. In air, the three lines all showed shorter roots and slightly but significantly longer coleoptiles compared with the wild type. After ethylene treatment, the lines exhibited much shorter and twisted roots, elongated mesocotyls, and long coleoptiles in comparison with the wild type (Fig. 4, E–G). These results indicated that *OsEIL2* overexpression conferred constitutive ethylene responses in air especially for roots but enhanced ethylene responses in ethylene for both roots and coleoptiles.

The expression of ethylene-inducible genes in shoots was examined in *OsEIL2* transgenic lines. *GERMIN-LIKE GENE*, *OsERF063*, *SHR5*, and *OsERF073* were found to be induced by ethylene in shoots of wild-type etiolated seedlings, but this induction was greatly blocked in *OsEIL2*-RNAi lines (Fig. 4H). Both *OsERF063* and *OsERF073* were found to be induced by ethylene in roots of wild-type seedlings. In roots of *OsEIL2*-RNAi lines, the ethylene induction of the two genes was moderately reduced when compared with that in the wild type (Supplemental Fig. S2). Furthermore, the expression levels of these genes were substantially enhanced in comparison with the wild type in shoots of air-grown etiolated overexpression seedlings (Fig. 4I). All these data indicate that *OsEIL2* is required for ethylene-induced expression of a subset of genes, especially in shoots.

We examined the expression of *MHZ6*/OsEIL1 and *OsEIL2* by real-time PCR and promoter-GUS analysis. The results showed that *MHZ6*/OsEIL1 and *OsEIL2* expression was detected in both roots and coleoptiles of etiolated seedlings (Supplemental Fig. S3, A and B). Their expression was also detected in organs from vegetative to reproductive stages and found to be more abundant in young leaves in plants (Supplemental Fig. S3, A–D).

For *OsEIL3* transgenic plants, three knockdown lines (1, 2, and 19) and three OX lines (17, 25, and 30) were selected, and they all exhibited almost similar phenotypes in both roots and coleoptiles compared with the wild type in the absence and presence of ethylene (Supplemental Fig. S4, A–C). Similar results were obtained in *oseil4* and three *OsEIL4*-OX lines (34, 47, and 57; Supplemental Fig. S5, A–C). These data suggest that *OsEIL3* and *OsEIL4* have no significant effects on the ethylene response in plants. Together with the results obtained with the OsEILs, we conclude that *MHZ6*/OsEIL1 and *OsEIL2* mainly mediated ethylene signaling in roots and coleoptiles of rice etiolated seedlings, respectively, whereas the other related proteins play no roles or very limited roles, if any, in ethylene signal transduction.



**Figure 4.** Knockdown of *OsEIL2* expression by RNAi confers strong ethylene insensitivity in coleoptiles, and overexpression of *OsEIL2* confers constitutive and enhanced ethylene responses in the absence and presence of ethylene, respectively. A, Expression levels of *OsEIL2* in *OsEIL2*-RNAi and *OsEIL2*-OX transgenic lines compared with the wild type (WT) by semiquantitative PCR. *OsACTIN1* was amplified as a loading control. B, Ethylene-response phenotypes of the wild type and *OsEIL2*-RNAi lines. The etiolated seedlings were grown in air or  $10 \mu\text{L L}^{-1}$  ethylene for 3 d. Bar = 10 mm. C, Length of roots in the wild type and *OsEIL2*-RNAi lines in response to ethylene. Values are means  $\pm$  SD ( $n \geq 30$ ). D, Length of coleoptiles in the wild type and *OsEIL2*-RNAi lines in response to ethylene. Values are means  $\pm$  SD ( $n \geq 30$ ). E, Ethylene-response phenotypes of the wild type and *OsEIL2*-OX lines. Details are as in B. F, Length of roots in the wild type and *OsEIL2*-OX lines in response to ethylene. Values are means  $\pm$  SD ( $n \geq 30$ ). G, Length of coleoptiles in the wild type and *OsEIL2*-OX lines in response to ethylene. Values are means  $\pm$  SD ( $n \geq 30$ ). Asterisks indicate significant differences from the corresponding controls at  $P < 0.01$ . H, Expression of ethylene-inducible genes in shoots of 2-d-old dark-grown wild-type and *OsEIL2*-RNAi plants with or without ethylene ( $10 \mu\text{L L}^{-1}$ ) for 8 h. Values are means  $\pm$  SD ( $n = 3$ ). I, Expression of ethylene-inducible genes in shoots of the wild type and three *OsEIL2*-OX lines. Values are means  $\pm$  SD ( $n = 3$ ).

### Simultaneous Knockdown of *MHZ6/OsEIL1* and *OsEIL2* Expression in Plants Confers Ethylene Insensitivity in Both Roots and Coleoptiles

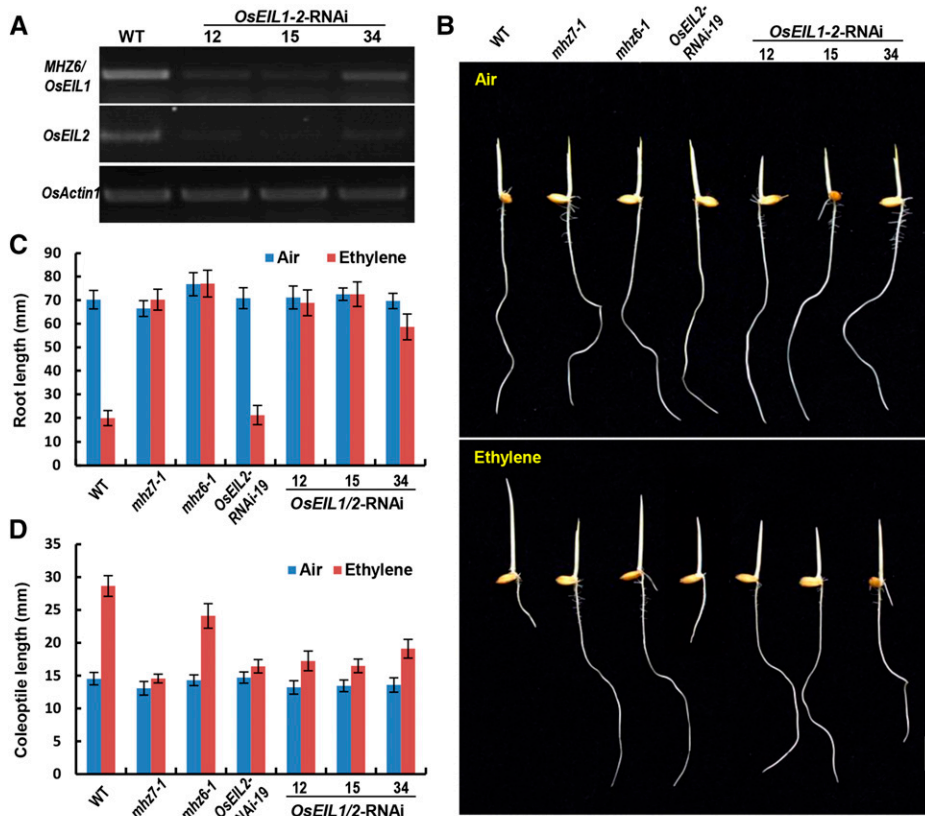
To further confirm this conclusion, we generated an RNAi construct that harbors a 407-bp fragment of the *MHZ6/OsEIL1* coding sequence in the reverse and forward directions and introduced it into rice plants. The sequence shares 50% identity with that of *OsEIL2*. Three *OsEIL1*-RNAi lines (12, 15, and 34), with both *MHZ6/OsEIL1* and *OsEIL2* knocked down, were obtained by semiquantitative PCR analysis (Fig. 5A). The three *OsEIL1*-RNAi lines showed largely insensitive phenotypes in both roots and coleoptiles, including longer roots and shorter coleoptiles, compared with the wild type in  $10 \mu\text{L L}^{-1}$  ethylene (Fig. 5, B–D). These results indicate that *MHZ6/OsEIL1* and *OsEIL2* may control the ethylene response collectively downstream of *OsEIN2* in rice.

### Mutation of *MHZ6/OsEIL1* and *OsEIN2/MHZ7* and Knockdown of *OsEIL2* Improve Salt Tolerance in Rice Plants

Previous studies in dicots have demonstrated that the components of the ethylene signaling pathway play important roles in plant salinity stress adaptation (Achard et al., 2006; Cao et al., 2006, 2007; Lei et al., 2011; Jiang et al., 2013; Li et al., 2014; Peng et al., 2014).

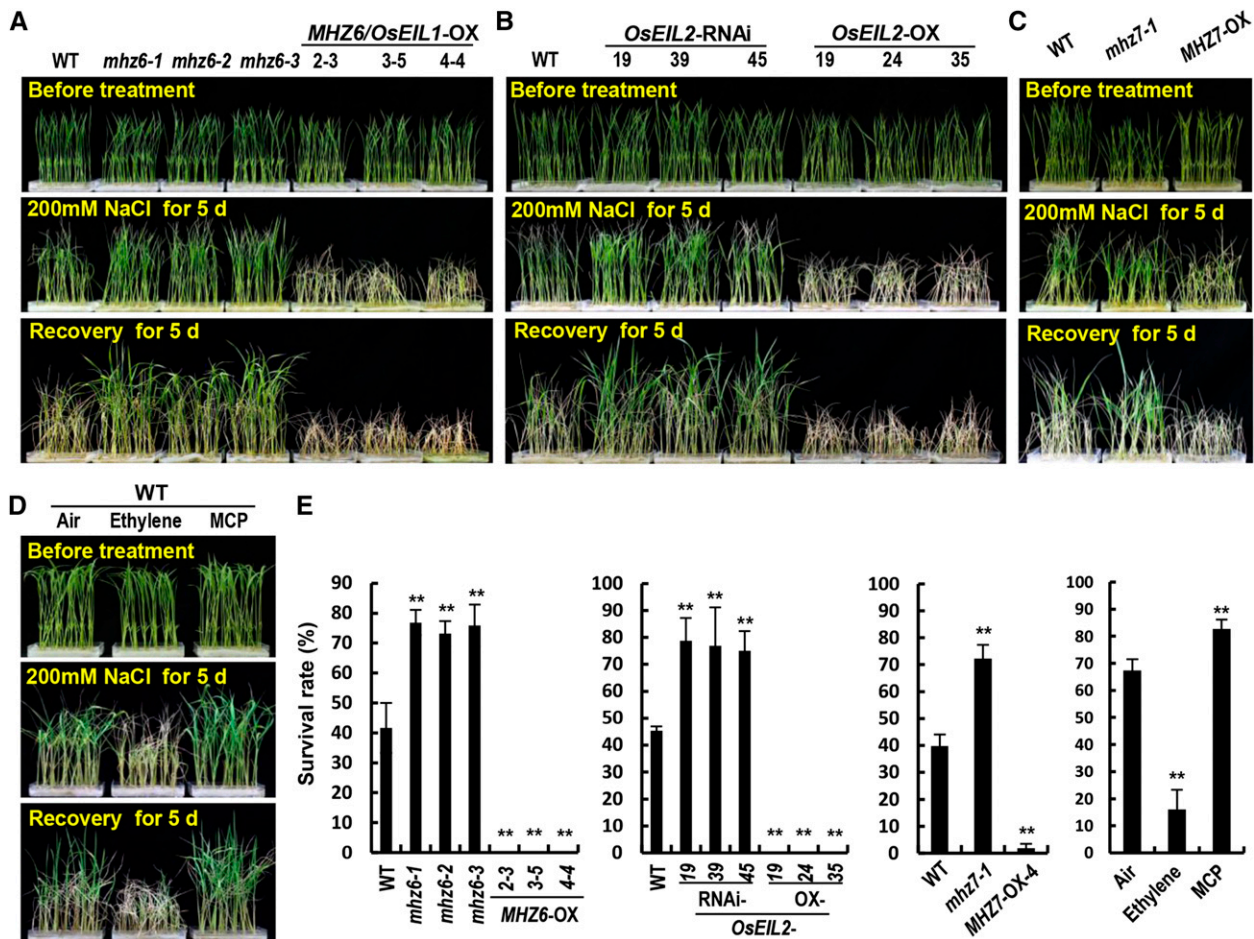
However, their roles in rice remain largely unknown. Thus, we examined whether *MHZ6/OsEIL1* and *OsEIL2* contribute to salt tolerance. Eight-day-old seedlings of the wild type, the three *mhz6* allelic mutants, and 9-d-old *MHZ6/OsEIL1*-OX lines were treated with 200 mM NaCl for 5 d and subsequently grown by regular hydroponic culture for 5 d. The mutant plants showed significantly higher survival rates compared with the wild type, whereas *MHZ6/OsEIL1*-OX seedlings were all wilted to death (Fig. 6, A and E). A similar performance was observed in *OsEIL2* knockdown and overexpression lines (Fig. 6, B and E). We also examined the salt tolerance of *mhz7/osein2-1* and *OsEIN2/MHZ7*-OX-4 plants obtained in our previous study (Ma et al., 2013). Compared with the wild type, more *mhz7-1* plants but fewer *OsEIN2/MHZ7*-OX-4 seedlings survived (Fig. 6, C and E). These results indicated that *MHZ6/OsEIL1*, *OsEIL2*, and *OsEIN2/MHZ7* play negative roles in salt stress tolerance in rice, in contrast to our and other previous studies in Arabidopsis in which mutants deficient for ethylene responses display salt hypersensitivity (Achard et al., 2006; Cao et al., 2007; Lei et al., 2011; Peng et al., 2014).

To corroborate the above results, we further investigated the effect of ethylene on salt stress. The wild-type seedlings of under 200 mM NaCl treatment were grown in air,  $10 \mu\text{L L}^{-1}$  ethylene, or  $5 \mu\text{L L}^{-1}$  1-MCP. Upon the treatment with 1-MCP, which blocks ethylene perception, the wild type displayed enhanced tolerance to salt compared with air-grown controls, as revealed from the



**Figure 5.** Knockdown of both *MHZ6/OsEIL1* and *OsEIL2* expression by RNAi (*OsEIL1*-2-RNAi) confers ethylene insensitivity in roots and coleoptiles. A, Expression levels of *MHZ6/OsEIL1* and *OsEIL2* in *OsEIL1*-2-RNAi transgenic lines compared with the wild type (WT) by semiquantitative PCR. *OsACTIN1* was amplified as a loading control. B, Ethylene-response phenotypes of the wild type, *osein2/mhz7-1*, *mhz6-1*, *OsEIL2*-RNAi-19, and three *OsEIL1*-2-RNAi lines. The etiolated seedlings were grown in air or  $10 \mu\text{L L}^{-1}$  ethylene for 3 d. Bar = 10 mm. C, Length of roots in the wild type, *osein2/mhz7-1*, *mhz6-1*, *OsEIL2*-RNAi-19, and three *OsEIL1*-2-RNAi lines in response to ethylene. Values are means  $\pm$  SD ( $n \geq 30$ ). D, Length of coleoptiles in the wild type, *osein2/mhz7-1*, *mhz6-1*, *OsEIL2*-RNAi-19, and three *OsEIL1*-2-RNAi lines in response to ethylene. Values are means  $\pm$  SD ( $n \geq 30$ ).





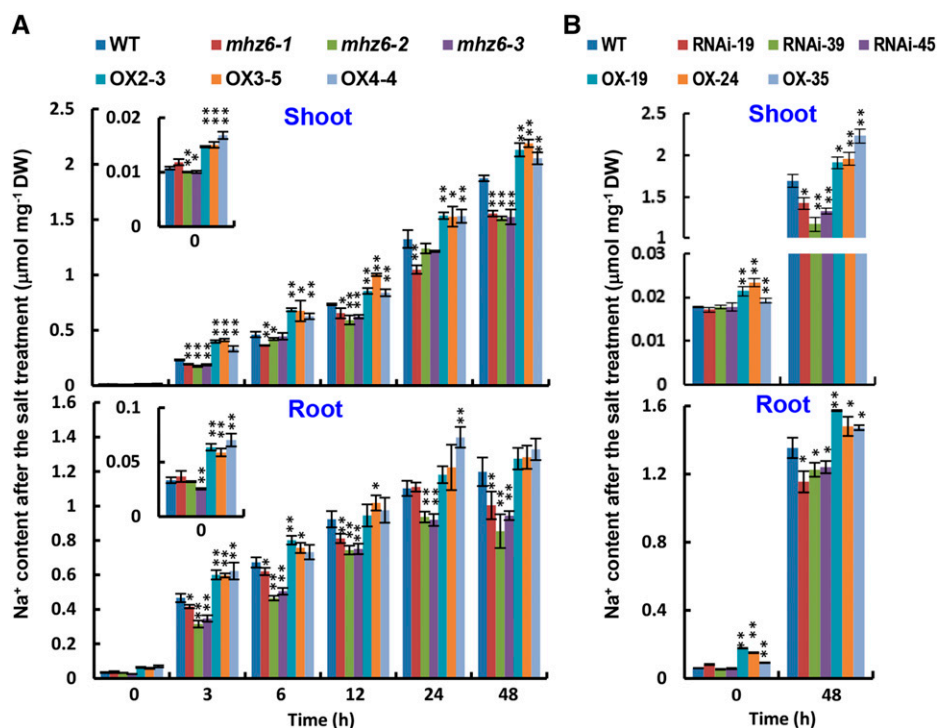
**Figure 6.** Performance of various plants under salt stress. A, Comparison of the wild type (WT), *mhz6* alleles, and *MHZ6/OsEIL1-OX* lines under salt stress. Eight- and 9-d-old seedlings were used as described in “Materials and Methods.” B, Comparison of the wild type, *OsEIL2-RNAi*, and *OsEIL2-OX* lines under salt stress. C, Comparison of the wild type, *mhz7-1*, and *MHZ7/OsEIL2-OX-4* lines under salt stress. D, Comparison of the wild type in air, 10  $\mu\text{L L}^{-1}$  ethylene, or 5  $\mu\text{L L}^{-1}$  1-MCP under salt stress. E, Survival rates after salt stress in A to D. Values are means  $\pm$  *sd* (*n* = 3). Asterisks indicate significant differences from the corresponding controls at *P* < 0.01.

higher survival rate. By comparison, the wild type under ethylene treatment showed a lower survival rate. These results indicate that ethylene treatment leads to salt sensitivity, whereas suppressed ethylene signaling improves salt tolerance in rice.

***MHZ6/OsEIL1* and *OsEIL2* Mutant/RNAi Plants Accumulate Less Na<sup>+</sup>, Whereas the OX Lines Accumulate More Na<sup>+</sup>, Than the Wild Type in Both Shoots and Roots under Salt Stress**

Salt tolerance in plants is the ultimate manifestation of several physiological processes. An important aspect is the avoidance of Na<sup>+</sup> accumulation. To investigate whether the performance of *mhz6* mutants and *MHZ6/OsEIL1-OX* lines under salt stress was related to Na<sup>+</sup> accumulation, 8-d-old hydroponically grown wild-type

plants, three allelic mutants, and 9-d-old *MHZ6/OsEIL1-OX* lines were subjected to treatment with 200 mM NaCl in Hoagland solution. After the stress, roots and shoots were harvested separately for analysis. The shoots of all the *mhz6* seedlings had substantially less Na<sup>+</sup> than the wild type during the salt stress, whereas the shoots of all *MHZ6/OsEIL1-OX* lines accumulated significantly more Na<sup>+</sup> than the wild type at all time points (Fig. 7A). Similarly, *mhz6* roots had significantly less Na<sup>+</sup> than the wild type, whereas roots of all OX lines accumulated more Na<sup>+</sup> than the wild type, especially at early hours of the salt stress (Fig. 7A). In contrast to Na<sup>+</sup>, levels of K<sup>+</sup> declined gradually in both shoots and roots of all plant lines tested during salt stress (Supplemental Fig. S6). It should be noted that the OX lines accumulated slightly more Na<sup>+</sup> and K<sup>+</sup> in shoots before treatment (Fig. 7A; Supplemental Fig. S6A). These results suggest



**Figure 7.** Na<sup>+</sup> content in shoot and root of various plants under salt stress. A, Comparison of Na<sup>+</sup> content in shoots and roots of the wild type (WT), *mhz6* alleles, and *MHZ6/OsEIL1*-OX lines under salt stress. Eight-day-old seedlings were subjected to Hoagland solution with 200 mM NaCl. The roots and shoots were harvested separately at the indicated times. Values are means  $\pm$  SD ( $n = 3$ ). Asterisks indicate significant differences from the corresponding controls at \*\*,  $P < 0.01$  and \*,  $P < 0.05$ . DW, Dry weight. B, Comparison of Na<sup>+</sup> content in shoots and roots of the wild type, *OsEIL2*-RNAi, and *OsEIL2*-OX seedlings under salt stress. Details are as in A.

that *MHZ6/OsEIL1* promotes Na<sup>+</sup> accumulation in rice seedlings under NaCl stress.

We also examined the Na<sup>+</sup> accumulation level in wild-type, *OsEIL2*-RNAi, and *OsEIL2*-OX lines. *OsEIL2* and *MHZ6/OsEIL1* had similar roles in Na<sup>+</sup> accumulation. Compared with the wild type, all *OsEIL2*-RNAi lines accumulated significantly less Na<sup>+</sup>, whereas all OX lines accumulated more Na<sup>+</sup> in both shoots and roots of seedlings treated with NaCl for 48 h (Fig. 7B). These results imply that the Na<sup>+</sup> level regulated by *OsEILs* may be one of the contributing mechanisms for salt-stress responses in rice.

#### *OsHKT* Genes Are Regulated by Ethylene and *MHZ6/OsEIL1* and *OsEIL2*

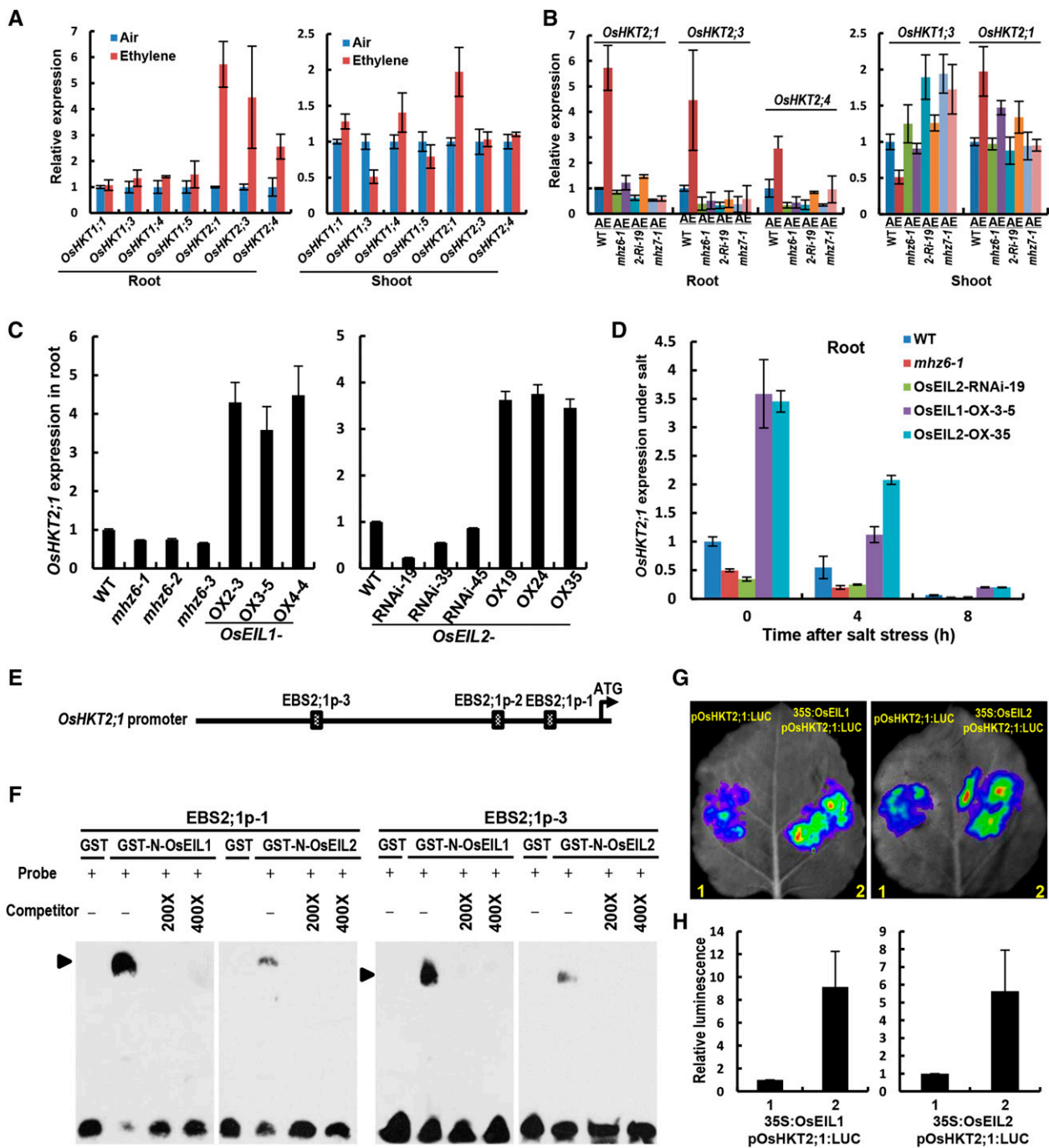
The results obtained above indicate that *MHZ6/OsEIL1* and *OsEIL2* positively regulate the Na<sup>+</sup> accumulation in rice seedlings under NaCl stress. In rice and Arabidopsis, there is evidence indicating that members of the HKT family function as Na<sup>+</sup> and Na<sup>+</sup>/K<sup>+</sup> transporters in controlling Na<sup>+</sup> accumulation (Garcia-deblás et al., 2003; Platten et al., 2006; Horie et al., 2009; Almeida et al., 2013). In Arabidopsis, there is only one *HKT* gene (Uozumi et al., 2000). In rice, the *HKT* gene family splits into two subfamilies, class I (*OsHKT1;1*, *OsHKT1;3*, *OsHKT1;4*, and *OsHKT1;5*) and class II (*OsHKT2;1*, *OsHKT2;3*, and *OsHKT2;4*; Platten et al., 2006). Therefore, we determined if ethylene signaling regulates the expression levels of *HKT* transporter genes.

In roots of wild-type seedlings, class II *HKT* genes, including *OsHKT2;1*, *OsHKT2;3*, and *OsHKT2;4*, were

induced to a higher level by ethylene (Fig. 8A, left), while class I *HKT* gene expression showed little change under ethylene treatment. In shoots, *OsHKT2;1* was induced to a level approximately 2-fold higher than the control, whereas *OsHKT1;3* expression was reduced by half in response to ethylene (Fig. 8A, right). The expression of other genes was only slightly changed (Fig. 8A, right). Further experiments showed that the ethylene induction of *OsHKT2;1* in roots and shoots, the induction of *OsHKT2;3* and *OsHKT2;4* in roots, and the suppression of *OsHKT1;3* in shoots were completely or largely blocked in seedlings of *mhz6-1*, *OsEIL2*-RNAi-19, and *mhz7-1* (Fig. 8B). These results indicate that ethylene regulates the expression of these *HKT* genes in an *MHZ7/OsEIN2*- and *OsEIL1/2*-dependent manner.

#### *MHZ6/OsEIL1* and *OsEIL2* Activate the Expression of *OsHKT2;1* through Direct Binding to Its Promoter

Previous studies have identified that *OsHKT2;1* and its homologous wheat (*Triticum aestivum*) protein TaHKT2;1 had similar roles in Na<sup>+</sup> uptake from the external medium (Laurie et al., 2002; Horie et al., 2007). Because ethylene induces *OsHKT2;1* expression via *OsEIL1/2* functions, we further examined the expression of *OsHKT2;1* in roots of the mutants/RNAi lines and OX lines of *MHZ6/OsEIL1* and *OsEIL2*. In comparison with the wild type, *OsHKT2;1* was slightly down-regulated in the three *mhz6* allelic mutants, whereas the expression was dramatically increased in the *MHZ6/OsEIL1*-OX lines (Fig. 8C, left). Similarly, *OsHKT2;1* expression was weakly



**Figure 8.** MHZ6/OsEIL1 and OsEIL2 directly activate the expression of *OsHKT2;1*. A, Expression of *OsHKT* family genes in both shoot and root of the wild type. Light-grown seedlings were treated with or without  $10 \mu\text{L L}^{-1}$  ethylene for 8 h. Values are means  $\pm$  SD ( $n = 3$ ). B, Expression of various *OsHKT*s in both shoot and root of wild-type (WT), *mhz6-1*, *OsEIL2-RNAi-19* (2-Ri-19), and *mhz7/osein2-1* plants. Details are as in A. C, Expression of *OsHKT2;1* in roots of MHZ6/*OsEIL1* and *OsEIL2* mutation/RNAi and OX lines. Details are as in A. D, Expression of *OsHKT2;1* in root of wild-type, *mhz6-1*, *OsEIL2-RNAi-19*, *OsEIL1-OX-3-5*, and *OsEIL2-OX-35* plants under salt stress. Details are as in A. E, Schematic diagrams of putative EBS in the promoter of *OsHKT2;1*. F, MHZ6/OsEIL1 and OsEIL2 proteins bind to the EBS-containing region of *OsHKT2;1*. Glutathione *S*-transferase (GST)-tagged MHZ6/OsEIL1 and OsEIL2 N-terminal fusion proteins were incubated with biotin-labeled DNA fragments (Probe). Competition for the biotin-labeled promoter region was done by adding an excess of unlabeled wild-type probe (Competitor). Two biological replicates and two technical replicates were performed with similar results. G, MHZ6/OsEIL1 and OsEIL2 activated the promoter activity of *OsHKT2;1* by transient expression in tobacco leaves. Five biological replicates were performed with similar results. H, Quantitative analysis of luminescence intensity for each comparison in G.

repressed in *OsEIL2*-RNAi plants but apparently induced in the *OsEIL2*-OX lines (Fig. 8C, right). We next examined the expression of *OsHKT2;1* in roots of various plants under 200 mM NaCl. *OsHKT2;1* was repressed in response to salt treatment in all the plants. However, compared with the wild type, the expression level of *OsHKT2;1* was still much higher in *MHZ6/OsEIL1*-OX-3-5 and *OsEIL2*-OX-35 plants but lower in *mhz6-1* and *OsEIL2*-RNAi-19 plants under salt treatment (Fig. 8D). These data indicate that *MHZ6/OsEIL1* and *OsEIL2* promote the expression of *OsHKT2;1* in roots and that salt stress reduces this gene expression.

We next analyzed the promoter region of *OsHKT2;1* and revealed three putative EIN3-binding sites (EBS-2;1-1, EBS-2;1-2, and EBS-2;1-3; Fig. 8E). To test the possibility that *MHZ6/OsEIL1* and *OsEIL2* directly bind to the promoter of *OsHKT2;1*, we carried out an electrophoresis mobility shift assay (EMSA) using the recombinant *MHZ6/OsEIL1* and *OsEIL2*-DBD. Two fragments of *OsHKT2;1* promoter sequence harboring EBS-2;1-1 or EBS-2;1-3 were specifically bound by *MHZ6/OsEIL1* or *OsEIL2*-DBD, respectively (Fig. 8F). These results indicate that both *MHZ6/OsEIL1* and *OsEIL2* directly bind to the *OsHKT2;1* promoter in vitro.

Using a tobacco transient expression assay system, we then tested whether *MHZ6/OsEIL1* and *OsEIL2* could activate the expression of *OsHKT2;1* in tobacco leaves. The 2,500-bp promoter sequence upstream from the ATG codon of *OsHKT2;1* was fused to the *LUCIFERASE* (*LUC*) reporter gene and cotransfected with each of the effector plasmids harboring 35S:*OsEIL1* or 35S:*OsEIL2* into the tobacco leaves. In contrast to the reporter vector control, the cotransfected effectors significantly increased the *OsHKT2;1* promoter-driven *LUC* activities (Fig. 8, G and H). These results demonstrate that *MHZ6/OsEIL1* and *OsEIL2* could activate the expression of *OsHKT2;1*. Together, these data indicate that *MHZ6/OsEIL1* and *OsEIL2* regulate the Na<sup>+</sup> uptake of plants through direct binding to the promoter and the activation of *OsHKT2;1* expression.

#### Effects of *MHZ6/OsEIL1* and *OsEIL2* on Plant Growth and Yield-Related Traits

Alterations of the components in ethylene signaling and ethylene biosynthesis could affect grain- and/or yield-related traits (Wuriyangan et al., 2009; Ma et al., 2013, 2014; Wang et al., 2013). Seedling growth from various plants in regular hydroponic culture for 7 d was examined under light. Compared with the wild type, the *mhz6* mutants had relatively straight and longer roots, whereas *MHZ6/OsEIL1*-OX plants had twisted and shorter roots and shorter shoots (Supplemental Fig. S7, A and B). Similar results were obtained from the *OsEIL2*-RNAi and *OsEIL2*-OX plants (Supplemental Fig. S7, C and D). These results suggest that light-grown *MHZ6/OsEIL1* and *OsEIL2* mutants/RNAi seedlings may have insensitive responses to the internal ethylene, whereas the OX plants have a constitutive ethylene response,

especially in roots during adaptive growth in hydroponic culture. The plant height of *mhz6* and *OsEIL1*-OX plants at maturity stage exhibited no significant difference in comparison with that of the wild type (data not shown). *OsEIL2*-RNAi plants also had similar height to wild-type plants (Supplemental Fig. S7, E and G). However, the *OsEIL2*-OX plants were obviously shorter than the wild-type plants (Supplemental Fig. S7, F and G). These results indicate that alteration of the *OsEIL1* and *OsEIL2* expression could affect the growth of seedlings and/or mature plants.

The grain size was examined in various plants. The *mhz6* mutants had a slight reduction in grain length, and all three mutants showed significant decreases in both grain width and thickness compared with wild-type grains (Supplemental Fig. S7, H and I). In *MHZ6/OsEIL1*-OX plants, grain length and width were increased significantly in all three lines compared with that in the wild type (Supplemental Fig. S7, H and I). These results indicate that *MHZ6/OsEIL1* mutation reduces grain size, whereas *MHZ6* overexpression increases grain size. The thousand-grain weight of various plants was further measured. The results showed that *mhz6* mutants had significant reductions, whereas the three OX plants had significant increases in this parameter (Supplemental Fig. S7I). The grain size and thousand-grain weight of two *OsEIL2*-OX plants (19 and 24) were smaller than those of the wild type, whereas the two parameters of the *OsEIL2*-RNAi plants showed some fluctuation compared with those of the wild type (Supplemental Fig. S7, J and K). These results indicate that the effects of *MHZ6/OsEIL1* and *OsEIL2* on grain size and weight are differential and that *MHZ6/OsEIL1* increases the grain size and thousand-grain weight in rice.

## DISCUSSION

In this study, we characterized a rice ethylene response-deficient mutant, *mhz6*, which showed ethylene insensitivity in roots. *MHZ6* encoded a homologous protein to EIN3, which is the master transcription factor in the ethylene signaling pathway of Arabidopsis, suggesting that ethylene signaling in rice may be conserved. However, our results reveal distinct roles for *MHZ6/OsEIL1* and its related gene *OsEIL2*, in contrast to Arabidopsis *EIN3/EIL1*, in both ethylene response and salt stress response. In rice, *MHZ6/OsEIL1* and *OsEIL2* regulate the ethylene responses of roots and coleoptiles, respectively, and both cause salt-sensitive responses. In Arabidopsis, *EIN3* and *EIL1* regulate similar ethylene responses in seedlings but confer salt tolerance.

#### *MHZ6/OsEIL1* and *OsEIL2* Regulate the Ethylene Response of Root and Coleoptile of Rice Etiolated Seedlings, Respectively

There are six members of the EIN3 family in Arabidopsis. Of these, *EIN3* and *EIL1* are the most related



proteins in ethylene-controlled transcriptional regulation (Chao et al., 1997; Alonso et al., 2003). Both *ein3* and *eil1* mutants exhibit incomplete ethylene insensitivity in etiolated seedlings (Alonso et al., 2003). Different from this phenomenon, the roots of our rice *mhz6/oseil1* seedlings displayed completely insensitive phenotypes when treated with ethylene (Fig. 1, A and B). By contrast, the coleoptile growth of *mhz6* seedlings was only slightly different from that of the wild type after ethylene treatment (Fig. 1, A and C). In the rice OsEIL family, OsEIL2s are closely related to MHZ6/OsEIL1. Silencing of *OsEIL2* leads to strong insensitivity in ethylene-promoted coleoptile elongation but almost normal ethylene sensitivity in root growth (Fig. 4, B–D). These results demonstrate that *MHZ6/OsEIL1* and *OsEIL2* mainly regulate the ethylene response in roots and coleoptiles, respectively, indicating their functional divergence in rice. This organ-specific functional divergence has not been observed in *EIN3* and *EIL1* of Arabidopsis, since both control similar ethylene responses in etiolated seedlings. The reason for this functional diversification of *MHZ6/OsEIL2* and *OsEIL2* is not clear. It is possible that this functional difference was acquired during rice domestication, since rice plants need a mechanism to adapt to the water environment. We have discovered that ethylene promotion of coleoptile elongation in etiolated seedlings is specific for rice among the monocotyledonous plants examined (Yang et al., 2015). Rice lives in water-saturated soil almost in its entire life cycle, and faster growing coleoptiles promoted by *OsEIL2* are conducive for rice to emerge from shallow waters, representing the mechanism adopted for rice adaptation in the water environment.

How *MHZ6/OsEIL1* and *OsEIL2* of the rice plants achieve their corresponding functions requires further investigation. In etiolated seedlings, both genes showed relatively higher expression in coleoptiles than in roots (Supplemental Fig. S2, A and B). Promoter-GUS analysis also reveals similar expression patterns (Supplemental Fig. S2, C and D). Therefore, the organ-specific functions of the two genes may not be due to differences in spatial expression patterns and/or intensities. It is possible that *MHZ6/OsEIL1* and *OsEIL2* may have different protein levels in roots and shoots. Moreover, the two proteins may activate different *ERF*-related genes for root inhibition or coleoptile promotion. In Arabidopsis, *EIN3* may regulate different *ERF* genes for stress and ethylene responses (Zhong et al., 2009; Zhang et al., 2011; Chang et al., 2013; Peng et al., 2014). Alternatively, the two proteins may also function through the direct regulation of downstream genes other than *ERFs*. Further identification of the target genes of *MHZ6/OsEIL1* and *OsEIL2* should shed light on the mechanisms involved.

It is interesting that *OsEIL2* promoted the expression of *MHZ6/OsEIL1* mildly in *OsEIL2*-OX transgenic plants (Supplemental Fig. S8A). Compared with the wild type, the expression of *MHZ6/OsEIL1* was

slightly inhibited in roots but apparently inhibited in shoots of *OsEIL2*-RNAi-19 (Supplemental Fig. S8A). Similarly, *MHZ6/OsEIL1* promoted *OsEIL2* expression especially in roots of *MHZ6/OsEIL1*-OX plants (Supplemental Fig. S8B). Consistently, the expression of *OsEIL2* had similar inhibition in roots and shoots of the *mhz6-1* mutant (Supplemental Fig. S8B). These results indicate that *MHZ6/OsEIL1* and *OsEIL2* may regulate each other at the transcript level. Considering that *MHZ6/OsEIL1* and *OsEIL2* mainly regulate ethylene responses in roots and coleoptiles/shoots, respectively, the mutual positive regulation of the two genes may be beneficial for the survival of the whole plants when only roots or shoots are exposed to different stresses.

We analyzed the ethylene-responsive genes in roots of *mhz7-2/osein2* and *mhz6-1* and in shoots of *mhz7-2/osein2* and *OsEIL2*-RNAi-19 compared with the wild type by RNA sequencing (RNA-seq; SRP041468). There are 1,046 ethylene-responsive genes in roots of the wild type, and *OsEIN2* and *MHZ6/OsEIL1* regulated 757 and 464 genes, respectively. Eighty-seven percent (404 genes) of the *MHZ6/OsEIL1*-regulated genes are also regulated by *OsEIN2* in roots (Supplemental Fig. S9A; Supplemental Table S1). In shoots, there are 5,112 ethylene-responsive genes. Among these, *OsEIN2* and *OsEIL2* regulated 4,815 and 4,515 genes, respectively, and the overlapping genes are 4,328, representing 95% of the *OsEIL2*-regulated genes (Supplemental Fig. S9B; Supplemental Table S2). These results further support that *MHZ6/OsEIL1* and *OsEIL2* mainly regulate the ethylene response of roots and coleoptiles, respectively, downstream of *OsEIN2*.

Knockdown of both *MHZ6/OsEIL1* and *OsEIL2* leads to insensitive phenotypes in both roots and coleoptiles of *OsEIL1-2*-RNAi plants (Fig. 5B). This phenomenon may be consistent with that of the Arabidopsis *ein3eil1* double mutants, which show complete ethylene insensitivity in both the triple response and repression of the *ctr1* mutation (Alonso et al., 2003). Therefore, similar to *EIN3* and *EIL1* from Arabidopsis, *MHZ6/OsEIL1* and *OsEIL2* could collectively mediate most aspects of seedling growth responses to ethylene in rice. Other members of the Arabidopsis *EIN3* family (*EIL2*–*EIL5*) may not play roles in ethylene signaling transduction. In this study, we found that the more distantly related members *OsEIL3* and *OsEIL4* also had transcriptional activities and were nuclear proteins (Supplemental Fig. S1, B and C). However, analyses of the RNAi/mutation and OX plants of *OsEIL3* and *OsEIL4* suggest that *OsEIL3* and *OsEIL4* likely have less effect on the ethylene response in etiolated seedlings (Supplemental Figs. S4, A–C, and S5, A–C).

Together, our results provide direct evidence that *MHZ6/OsEIL1* and *OsEIL2* mediate ethylene signaling in rice seedlings by spatially regulating the ethylene response of root and coleoptile, respectively.

### MHZ6/OsEIL1 and OsEIL2 Negatively Regulate the Salt Tolerance of Rice Seedlings via the Direct Transcriptional Regulation of *OsHKT2;1* in Roots

Our previous studies in the ethylene receptor NTHK1 in tobacco and EIN2 and EIN3/EIL1 in Arabidopsis have demonstrated that ethylene plays a positive role in salt stress tolerance in dicotyledonous plants (Cao et al., 2006, 2007; Chen et al., 2009; Lei et al., 2011). An ankyrin domain protein, NEIP2, that interacted with NTHK1 is also required for salt-stress tolerance in tobacco plants (Cao et al., 2015). Recently, studies on EIN3/EIL1 function in salt stress have further supported the above view (Peng et al., 2014). Increased *in vivo* ethylene production via loss of ETO1 function also confers salt tolerance in Arabidopsis (Jiang et al., 2013). At present, we found that, in contrast to the positive roles of EIN3 and EIL1 in Arabidopsis, MHZ6/OsEIL1 and OsEIL2 play negative roles in the salt tolerance of rice seedlings. Rice seedlings with ethylene treatment displayed reduced salt tolerance, whereas blocking ethylene perception with 1-MCP leads to enhanced tolerance of rice seedlings (Fig. 6D). These results further support that increased ethylene or enhanced ethylene signaling decreases the salt tolerance of rice seedlings. Consistent with this, the rice receptor-like kinase SALT INTOLERANCE1, which promotes ethylene production in rice, causes salt sensitivity (Li et al., 2014). Similarly, down-regulating the ethylene biosynthetic pathway by knocking down *1-aminocyclopropane-1-carboxylic acid synthase3* can improve the performance and grain yield of maize (*Zea mays*) under abiotic stress (Habben et al., 2014).

When plants are grown in conditions of high salt, the avoidance of Na<sup>+</sup> uptake is an important aspect of salt tolerance (Munns and Tester, 2008). In Arabidopsis, *EIN3ox* seedlings accumulated less Na<sup>+</sup>, whereas *ein3-leil1-1* seedlings had higher Na<sup>+</sup> content compared with the wild type (Peng et al., 2014). The plants lacking ETO1 function accumulated much less shoot Na<sup>+</sup> than the wild type (Jiang et al., 2013). On the contrary, our observations indicate that, under high-salt conditions, loss of MHZ6/OsEIL1 and OsEIL2 functions reduced Na<sup>+</sup> accumulation, whereas overexpression of them increased Na<sup>+</sup> accumulation in plants (Fig. 7), suggesting that MHZ6/OsEIL1 and OsEIL2 promote Na<sup>+</sup> accumulation and hence cause salt-sensitive responses in rice.

It should be noted that the ion content differences between mutant/RNAi or OX plants and wild-type plants are not that large in this study (Fig. 7), very similar to the cases in rice and wheat of other studies (Laurie et al., 2002; Ren et al., 2005). However, in Arabidopsis, the Na<sup>+</sup> contents are strikingly different in the plants compared (Jiang et al., 2013; Peng et al., 2014). It is possible that the Na<sup>+</sup> accumulation is differential in different plant species. Alternatively, in rice, ethylene signaling may control only a limited portion of Na<sup>+</sup> accumulation. Other possibilities could not be excluded.

We further elucidated the specific downstream targets of the ethylene signaling cascade controlling the Na<sup>+</sup> content in rice and found that MHZ6/OsEIL1 and OsEIL2 positively regulate the expression of *OsHKT2;1* in roots by directly binding to its promoter. Ethylene also induced *OsHKT2;1* expression via *MHZ7/OsEIN2* and *MHZ6/OsEIL1* and *OsEIL2* functions. *OsHKT2;1* is classified as a member of HKT subfamily II, which appears to be specific to monocotyledonous plants (Platten et al., 2006; Almeida et al., 2013). *OsHKT2;1* mediates Na<sup>+</sup> import in the presence of Na<sup>+</sup> in the millimolar range (Jabnour et al., 2009). In comparison with the wild type, the knockout mutants of *OsHKT2;1* have large reductions in Na<sup>+</sup> uptake (Horie et al., 2007). In addition to *OsHKT2;1*, two homologous genes, *TaHKT2;1* and *HvHKT2;1*, of HKT subfamily II have been characterized in wheat and barley (*Hordeum vulgare*; Gassmann et al., 1996; Wang et al., 1998; Rubio et al., 1999; Buschmann et al., 2000; Gollmack et al., 2002; Laurie et al., 2002; Haro et al., 2005; Bañuelos et al., 2008; Mian et al., 2011). The antisense repression of *TaHKT2;1* expression resulted in a decrease in Na<sup>+</sup> uptake by the roots and increased salt tolerance in wheat (Laurie et al., 2002). *HvHKT2;1* overexpression increases Na<sup>+</sup> content in plants grown at high external Na<sup>+</sup> concentrations in barley (Mian et al., 2011). Together, these results reveal that the members of class II HKT have a role in Na<sup>+</sup> uptake from the external medium. Moreover, *OsHKT2;1*, *TaHKT2;1*, and *HvHKT2;1* are expressed in root periphery cells, which agrees with their functions in ion uptake from the external medium (Schachtman and Schroeder, 1994; Gollmack et al., 2002; Jabnour et al., 2009). Therefore, the MHZ6/OsEIL1- and OsEIL2-mediated regulation of *OsHKT2;1* in roots may contribute to the Na<sup>+</sup> accumulation and salt sensitivity of rice seedlings under salt stress.

Interestingly, it seems likely that HKT proteins evolved rapidly. The Arabidopsis genome contains a single *HKT* gene, whereas rice contains up to nine *HKT* genes (depending on variety). The main role of *AtHKT1;1* is to avoid the accumulation of excessive Na<sup>+</sup> in the shoots of Arabidopsis (Berthomieu et al., 2003; Sunarpi et al., 2005; Møller et al., 2009). In rice, ethylene and MHZ6/OsEIL1- and OsEIL2-regulated *OsHKT2;1* have distinct roles in Na<sup>+</sup> uptake from the external medium. The ethylene signaling-driven Na<sup>+</sup> uptake may be useful for rice to adapt to the environment of high water potential by maintaining the ion homeostasis of plant cells. This mechanism is essential for the survival of rice plants under normal water conditions. However, when exposed to high salinity, the high *OsHKT2;1* expression induced by the enhanced ethylene level and *MHZ6/OsEIL1* and *OsEIL2* expression result in excessive Na<sup>+</sup> accumulation and, hence, salt sensitivity of plants. It should be noted that salt treatment represses *OsHKT2;1* expression in rice, suggesting that rice plants may also try to avoid excessive Na<sup>+</sup> uptake through the inhibition of *OsHKT2;1* expression under high-salinity conditions. It will be interesting to test the performance of the plants with altered ethylene

signaling under long-term salt stress conditions in the field.

Besides *OsHKT2;1*, ethylene also affected the expression of some other members of the *OsHKT* family (Fig. 8, A and B). *MHZ6/OsEIL1* and *OsEIL2* positively regulated *OsHKT2;3* and *OsHKT2;4* in roots but negatively regulated *OsHKT1;3* in shoots (Supplemental Fig. S10, A–C), most likely through direct binding to their promoters (Supplemental Fig. S10, D–G). *OsHKT2;3* and *OsHKT2;4*, members of *HKT* subfamily II like *OsHKT2;1*, were marginally expressed in the roots (Lan et al., 2010; Horie et al., 2011). In *Xenopus laevis* oocytes, *OsHKT1;3* mediated both inward and outward  $\text{Na}^+$  currents with weak inward rectification, although yeast cells expressing *OsHKT1;3* did not mediate any type of transport (Garcia-deblás et al., 2003; Jabnourne et al., 2009). The function of *OsHKT1;3* in rice has not been studied yet. Another subfamily I member, *OsHKT1;5*, corresponding to *Streptomyces lividans*  $\text{K}^+$  Channel1, is an  $\text{Na}^+$ -selective transporter and positively regulated  $\text{K}^+/\text{Na}^+$  homeostasis under salt stress (Ren et al., 2005). It will be interesting to identify the functions of the above *OsHKT* transporters in plants to determine whether *MHZ6/OsEIL1* and *OsEIL2* regulation of their expression is involved in the salt stress responses of rice seedlings.

Taken together, different from the positive roles of ethylene and EIN3/EIL1 in Arabidopsis, our results prove that *MHZ6/OsEIL1* and *OsEIL2* negatively regulate the salt tolerance of rice seedlings by direct regulation of *OsHKT2;1* expression and  $\text{Na}^+$  uptake. Understanding the molecular mechanism of the interaction between ethylene and salt stress in rice will provide insights for engineering salt tolerance in important crops.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Rice (*Oryza sativa* ssp. *japonica* 'Nipponbare') was used as the wild type in this study. All plants were grown in the paddy field of the Experimental Station of the Institute of Genetics and Developmental Biology in Beijing from May to October of each year.

### Ethylene Treatment

For the ethylene-response assay, seeds were soaked at 37°C for 2 d, and the germinated seeds were placed on stainless nets with a water level below the seeds. Ethylene gas was injected into the boxes using a syringe. The seedlings were grown at 28°C in the dark for 3 d (Ma et al., 2013). The length of roots and coleoptiles was measured.

For ethylene treatment of etiolated/green seedlings, germinated seeds were placed on stainless nets for 2/3 d in air and then treated with  $10 \mu\text{L L}^{-1}$  ethylene for 8 h at 28°C in dark/constant light. The roots and coleoptiles were harvested for total RNA isolation.

### Molecular Cloning of *MHZ6*

To map the *MHZ6* locus, the mutant *mhz6-1* was crossed with the *indica* cv 93-11 MH63, cv ZF802, and cv TN1. For rough mapping, we used a DNA pool generated from 15 mutant individuals selected from the F2 population derived from a cross between *mhz6-1* and MH63. For fine mapping, a total of 900 mutant

individuals selected from the four F2 populations were used. The *MHZ6* locus was mapped to a 68-kb region (3-11.76, 5'-GCCAAGACGCCAACCAAAA-3' and 5'-GGTGGAGGGGAGTGGGAT-3'; 3-11.83, 5'-CCCTGATGTTTCCCCTTTTA-3' and 5'-TATCAATTTTGATCCCCTCAT-3'), which contains 13 genes. The candidate gene was finally determined by DNA sequencing of all the genes in this region.

To confirm the mutation in *mhz6*, DNA fragment-length polymorphism between the wild type and the mutant was examined. Considering the duplication of the *MHZ6* sequence, nested PCR was used. The primers are listed in Supplemental Table S1.

### Gene Expression Analysis by Semiquantitative PCR or Real-Time PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen). After eliminating DNA contamination by DNase I (TaKaRa), the complementary DNAs were synthesized using the cDNA Synthesis Kit (Moloney murine leukemia virus version; TaKaRa) and then subjected to semiquantitative PCR or real-time quantitative PCR. *OsACTIN1* and/or *OsACTIN2* were used as the internal controls. The primers used are listed in Supplemental Table S3.

### Vector Construction and Plant Transformation

For complementation, the pCAMBIA2300 vector (pMHZ6C) contained the *MHZ6* genomic sequence (1,923 bp), the upstream 2,588-bp sequence of the *MHZ6* ATG (promoter), and the downstream 2,117-bp sequence of the *MHZ6* TGA. This full sequence (6,628 bp) was cloned by adding two separate regions: the first region (1–3,303 bp) with *EcoRI* and *KpnI* sites, and the second region (3,304–6,628bp) with *KpnI* and *Sse8387I*.

For the *OsEIL* OX vectors, taking *MHZ6-OX* vector as an example, the full coding sequence (1,923 bp) and 3' untranslated region (43 bp) were inserted into *BamHI/XbaI*-digested pCAMBIA2300-35S vector to generate the *MHZ6-OX* construct.

For the promoter:GUS constructs, 2,588- and 2,618-bp fragments upstream from *MHZ6/OsEIL1* ATG and *OsEIL2* ATG were amplified for *MHZ6/OsEIL1*:GUS and *OsEIL2*:GUS, respectively. The fragments were then inserted into pCAMBIA2300-GUS vector.

For the *OsEIL* RNAi vectors, taking *OsEIL2*-RNAi vector as an example, two copies of the 484-bp specific 3' coding sequence were repeatedly inserted in opposing orientations into pUCCRNAi to form a hairpin, which was then cloned into pCAMBIA2300-pAct1. The primers used for the constructs are listed in Supplemental Table S3.

All the vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 through electroporation, and the resulting strains were used to transform the *japonica* rice cv Nipponbare. Rice transformation was performed as described previously (Wuriyangan et al., 2009).

### Transactivation Analysis

The transactivation activity was examined in the Arabidopsis (*Arabidopsis thaliana*) protoplast system. The reporter was a plasmid harboring the firefly *LUC* gene, which was controlled by a modified 35S promoter with 5× the upstream activating sequence in it. *OsEIL* genes were fused to the GAL4 (yeast transcription activator Gal4) DBD-coding sequence and constructed into pRT107 to generate effector plasmid pRTBD-*OsEIL*s. The fusion genes were under the control of the 35S promoter. pRT107 vector containing the binding domain sequence and the 375BD-VP16 fusion sequence were used as negative and positive controls, respectively. A pPTRL plasmid that contained a cauliflower mosaic virus 35S promoter and Renilla LUC was used as an internal control (Ohta et al., 2000). The primers used for the constructs are listed in Supplemental Table S3.

### Subcellular Localization Assays

For the detection of subcellular localization of *OsEIL*s, the full-length *OsEIL* complementary DNA sequence was amplified and cloned into the pBI221-GFP or pJIT163-hGFP vector in which the GFP-coding sequence was fused in frame to the 3' end of the *OsEIL* gene sequence. Primers are listed in Supplemental Table S3. The constructs were transformed into Arabidopsis protoplasts by polyethylene glycol-mediated transformation (Yoo et al.,

2007). After 20 h of incubation at 28°C, GFP fluorescence was observed with a confocal laser scanning microscope (Leica TCS SP5).

## NaCl Treatment

All seeds were surface sterilized and germinated in water for 2 d, placed on the petri dishes (10 × 10 cm) with two layers of gauze, incubated in Hoagland solution (pH 6) for 8 or 9 d, and treated with 200 mM NaCl-containing Hoagland solution for another 5 d. After 5 d of recovery, the number of surviving seedlings was counted. All plants were grown under white fluorescent light (600 photons m<sup>-2</sup> s<sup>-1</sup>, 14 h of light/10 h of dark) at 28°C/26°C and 75% relative humidity. These experiments were repeated at least three times with similar results.

## Measurement of Na<sup>+</sup> and K<sup>+</sup> Concentrations

Ten-day-old seedlings grown in Hoagland solution were supplemented with 200 mM NaCl and grown for 2 d. Shoots and roots were harvested at the indicated times. Subsequently, shoots and roots were collected, washed six times with distilled water to eliminate the attached metal ions on the surface, and dried at 80°C for 72 h. Samples were then weighed and digested as follows: 15 mL of nitric acid, 160°C for 1 h; 2 mL of hydrogen peroxide, 20 min; 5 mL of nitric acid, 160°C for 30 min; and 1 mL of hydrogen peroxide, 10 min. Na<sup>+</sup> and K<sup>+</sup> contents were measured using an inductively coupled plasma optical emission spectrometer (Perkin Elmer) following a method described previously (Yuan et al., 2008). The means and SD were calculated from three biological replications.

## EMSA

To construct plasmids for the expression of N-terminal OsEIL1 (amino acids 1–350) and OsEIL2 (amino acids 1–354) regions in *Escherichia coli* BL21, DNA fragments corresponding to the two regions were obtained and inserted into the pGEX-6p-1 vector (Amersham). Purification of the fusion protein was conducted according to the Glutathione Sepharose 4B (GE) handbook.

Single-stranded complementary oligonucleotide fragments corresponding to regions of OsHKT promoters (Supplemental Table S3) harboring the EBS elements were synthesized (Invitrogen) and biotinylated using the Biotin 3'-end DNA Labeling Kit (Thermo Fisher Scientific). Biotinylated and unlabeled complementary oligonucleotide pairs were annealed to make double-stranded biotin-labeled probes and competitors by mixing together equal amounts, boiling for 5 min, and cooling slowly overnight. EMSA reaction solutions were prepared according to the manufacturer's protocol (LightShift Chemiluminescent EMSA Kit; Thermo Fisher Scientific). Reaction solutions were incubated for 20 min at room temperature. The protein-probe mixture was separated on a 6% polyacrylamide native gel and transferred to a nylon membrane (GE). After UV light cross-linking, the DNA on the membrane was detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific).

## Transactivation of the Target Promoter in Tobacco Leaves

The 2.5-kb sequences upstream from the ATG codons of *OsHKTs* were inserted into pGWB435 to generate promoter:LUC reporter constructs using Gateway technology (Invitrogen). The reporter plasmid and the constructs containing 35S:OsEIL1 and 35S:OsEIL2 were transformed into *A. tumefaciens* strain EHA105. The strains were incubated in Luria-Bertani medium and finally resuspended in infiltration buffer (10 mM MES, 0.2 mM acetosyringone, and 10 mM MgCl<sub>2</sub>) to an ultimate concentration of optical density at 600 nm = 1. Equal amounts of different combined bacterial suspensions were infiltrated into the young leaves of 5-week-old tobacco (*Nicotiana tabacum*) plants using a needleless syringe. After infiltration, the plants were grown first in the dark for 12 h and then with 16 h of light/8 h of dark for 48 h at 24°C before CCD imaging. The leaves were sprayed with 100 μM luciferin (Promega) and placed in the dark for 5 min. LUC activity was observed with a low-light cooled CCD imaging apparatus (iXon; Andor Technology). Experiments were performed with three independent biological replicates.

## Library Preparation and Bioinformatics Analysis

Two-day-old etiolated seedlings of the wild type, *mhz7-2/osein2* (Ma et al., 2013), *mhz6-1*, and *OsEIL2*-RNAi-19 were treated with or without 10 μL L<sup>-1</sup>

ethylene for 8 h, and the RNA was isolated. The mRNA was purified from 2 μg of total RNA using the Dynabeads mRNA Purification Kit (Invitrogen) and then subjected to RNA-seq library construction for the transcriptome experiments using the Ultra RNA Library Prep Kit (New England Biolabs) according to the manufacturer's protocol. Multiplex paired-end adapters (New England Biolabs) were used to multiplex libraries. The RNA-seq libraries were quantified using Bioanalyzer (Agilent) and then sequenced (paired end, 100 bp each) in the Illumina genome analyzer (HiSeq 2000). After removing adaptor and low-quality reads, clean reads were mapped to the rice genome MSU7.0 using TopHat and analyzed using Cufflinks according to Trapnell et al. (2012) with a little modification, which includes the Poisson dispersion model of fragments being used to conduct statistical analysis (false discovery rate < 0.05) and ethylene-responsive genes being identified by fragments per kilobase per million reads requiring more than 1.5-fold change between two samples.

Sequence data from this article can be found in the MSU7.0 database under the following accession numbers: *MHZ6/OsEIL1*, Os03g20790; *OsEIL2*, Os07g48630; *OsEIL3*, Os09g31400; *OsEIL4*, Os08g39830; *OsEIL5*, Os02g36510; *OsEIL6*, Os04g38400; *SHR5*, Os08g10310; *GERMIN-LIKE*, Os08g13440; *OsERF063*, Os09g11480; *OsERF073*, Os09g11460; *OsERF002*, Os06g08340; *OsRAP2.8*, Os11g05740; *OsACTIN1*, Os03g50885; *OsACTIN2*, Os10g36650; *OsHKT1;1*, Os04g51820; *OsHKT1;3*, Os02g07830; *OsHKT1;4*, Os04g51830; *OsHKT1;5*, Os01g20160; *OsHKT2;1*, Os06g48810; *OsHKT2;3*, Os01g34850; and *OsHKT2;4*, Os06g48800.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Transactivation analysis and subcellular localization of OsEILs.

**Supplemental Figure S2.** Expression of ethylene-inducible genes in roots of wild-type and *OsEIL2*-RNAi lines.

**Supplemental Figure S3.** Expression patterns of *MHZ6/OsEIL1* and *OsEIL2*.

**Supplemental Figure S4.** Knockdown and overexpression of *OsEIL3* in rice showed a similar response to ethylene compared with the wild type.

**Supplemental Figure S5.** Mutation and overexpression of *OsEIL4* in rice showed a similar response to ethylene compared with the wild type.

**Supplemental Figure S6.** K<sup>+</sup> content in shoots and roots of various plants under salt stress.

**Supplemental Figure S7.** Phenotype analysis of mutants and transgenic lines of *MHZ6/OsEIL1* and *OsEIL2*.

**Supplemental Figure S8.** Expression of *OsEIL1* and *OsEIL2* in various plants.

**Supplemental Figure S9.** Venn diagrams showing the overlaps among the ethylene-responsive genes regulated by *OsEIL1*, *OsEIL2* and *OsEIN2*.

**Supplemental Figure S10.** *MHZ6/OsEIL1* and *OsEIL2* affect the expression of *OsHKTs* by directly binding to their promoter.

**Supplemental Table S1.** Ethylene-responsive genes regulated by *OsEIN2* and *OsEIL1* analyzed by RNA-seq in roots of etiolated seedlings.

**Supplemental Table S2.** Ethylene-responsive genes regulated by *OsEIN2* and *OsEIL2* analyzed by RNA-seq in shoots of etiolated seedlings.

**Supplemental Table S3.** Primers and oligonucleotides used in this study.

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