Ethylene Responses in Rice Roots and Coleoptiles Are Differentially Regulated by a Carotenoid Isomerase-Mediated Abscisic Acid Pathway

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Ethylene and abscisic acid (ABA) act synergistically or antagonistically to regulate plant growth and development. ABA is derived from the carotenoid biosynthesis pathway. Here, we analyzed the interplay among ethylene, carotenoid biogenesis, and ABA in rice (Oryza sativa) using the rice ethylene response mutant mhz5, which displays a reduced ethylene response in roots but an enhanced ethylene response in coleoptiles. We found that MHZ5 encodes a carotenoid isomerase and that the mutation in mhz5 blocks carotenoid biosynthesis, reduces ABA accumulation, and promotes ethylene production in etiolated seedlings. ABA can largely rescue the ethylene response of the mhz5 mutant. Ethylene induces MHZ5 expression, the production of neoxanthin, an ABA biosynthesis precursor, and ABA accumulation in roots. MHZ5 overexpression results in enhanced ethylene sensitivity in roots and reduced ethylene sensitivity in coleoptiles. Mutation or overexpression of MHZ5 also alters the expression of ethylene-responsive genes. Genetic studies revealed that the MHZ5-mediated ABA pathway acts downstream of ethylene signaling to inhibit root growth. The MHZ5-mediated ABA pathway likely acts upstream but negatively regulates ethylene signaling to control coleoptile growth. Our study reveals novel interactions among ethylene, carotenogenesis, and ABA and provides insight into improvements in agronomic traits and adaptive growth through the manipulation of these pathways in rice.

INTRODUCTION

Ethylene is synthesized from methionine by three well-defined enzymatic reactions (Lin et al., 2009). The biosynthesis pathway of ethylene is regulated via the 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) enzymes at either the transcriptional or posttranslational levels (Argueso et al., 2007; Lyzenga and Stone, 2012; Lyzenga et al., 2012). Ethylene is perceived by a multimember family of membrane-bound receptors that possess sequence similarity with the bacterial two-component His kinase (Hall et al., 2007). Then, the ethylene signal is transmitted via a simple signal cascade that comprises CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), ETHYLENE INSENSITIVE2 (EIN2), EIN3, and/or EIN3-LIKE1 (An et al., 2010; Mayerhofer et al., 2012; Qiao et al., 2012; Merchante et al., 2013). In addition to the linear pathway, several components, including REVERSION TO ETHYLENE SENSITIVITY1 (RTE1) (Resnick et al., 2006; Dong et al., 2010), EIN3 Binding F-box 1 and 2 (Guo and Ecker, 2003), EIN2-TARGETING PROTEIN1 (ETP1) and ETP2, also play important regulatory roles in the ethylene response (Qiao et al., 2009).

Unlike ethylene gas, abscisic acid (ABA) has more complicated biosynthesis and signaling pathways (Nambara and Marion-Poll, 2005), comprising a core signaling pathway (Cutler et al., 2010; Finkelstein, 2013), catabolic regulation (Nambara and Marion-Poll, 2005; Xu et al., 2013), and requirement for a series of transporters (Kumomori et al., 2010; Kanno et al., 2012). Because ABA is produced through the cleavage of carotenoid precursors (xanthophylls) (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005), the supply of these carotenoid precursors has an important effect on ABA production.

Plant carotenoids are tetraterpenes that are synthesized in plastids and play different roles in plants. In photosynthetic plant
tissues, carotenoids contribute to light harvesting, photoprotection (Niyogi, 1999; Dall’Osto et al., 2007; Wei et al., 2010; Ballotari et al., 2014), and flower and fruit color (Hirschberg, 2001; Ruiz-Sola and Rodríguez-Concepción, 2012). Carotenoids are also present in nonphotosynthetic plastids, such as the etioplasts of dark-grown seedlings and the leucoplasts of roots, where they provide nutritional value (Howitt and Pogson, 2006).

The first committed step of the carotenoid pathway is the condensation of two molecules of geranylgeranylphosphate to form phytoene, a reaction catalyzed by phytoene synthase (PSY) (Rodríguez-Villalón et al., 2009). Phytoene then undergoes four consecutive desaturation reactions, leading to the formation of lycopene in reactions catalyzed by phytoene desaturase and \( \zeta \)-carotene desaturase. The production of all-trans-lycopene also requires Z-ISO (Chen et al., 2010) and carotenoid isomerase (CRTISO) (Isaacson et al., 2002; Park et al., 2002; Isaacson et al., 2004). Lycopene can be further converted into \( \alpha \)-carotene and/or \( \beta \)-carotene, which are catalyzed by \( \alpha \)-cyclases and \( \beta \)-cyclases, respectively (Cunningham et al., 1996). \( \beta \)-Carotene, which serves as a precursor for the plant hormone strigolactone (SL), can be further metabolized to \( \beta \)-\( \beta \)-xanthophylls such as zeaxanthin (Nambara and Marion-Poll, 2005; Xie et al., 2010). ABA is produced from violaxanthin or neoxanthin through several enzymatic reactions, including 9-cis-epoxycarotenoid dioxygenase (NCED), neoxanthin-deficient 1, alcohol dehydrogenase (ABA2)/short-chain dehydrogenase/reductase, abscisic aldehyde oxidase (AAO3), and sulfated molybdenum cofactor sulfurate (ABA3) (Nambara and Marion-Poll, 2005; Finkelstein, 2013; Neuman et al., 2014).

Crosstalk between ethylene and ABA occurs at multiple levels. One of these interactions is at the level of biosynthesis. Endogenous ABA limits ethylene production (Tal, 1979; Rakitina et al., 1994; LeNoble et al., 2004), and ethylene can inhibit ABA biosynthesis (Hoffmann-Benning and Kende, 1992). Previous studies have suggested that both ethylene and ABA can inhibit root growth (Vandenbussche and Van Der Straeten, 2007; Arc et al., 2013). In Arabidopsis thaliana, the etr1-1 and ein2 roots are resistant to both ethylene and ABA, whereas the roots of the ABA-resistant mutant abf1-1 and the ABA-deficient mutant aba2 have normal ethylene responses. This suggests that the ABA inhibition of root growth requires a functional ethylene signaling pathway but that the ethylene inhibition of root growth is ABA independent (Beaudoin et al., 2000; Ghaysemian et al., 2000; Cheng et al., 2009). Recent studies have indicated that ABA mediates root growth by promoting ethylene biosynthesis in Arabidopsis (Luo et al., 2014). However, the interaction between ethylene and ABA in the regulation of the rice (Oryza sativa) ethylene response is largely unclear.

Rice is an extremely important cereal crop worldwide that is grown under semiaquatic, hypoxic conditions. Rice plants have evolved elaborate mechanisms to adapt to hypoxia stress, including coleoptile elongation, adventitious root formation, aerenchyma development, and enhanced or repressed shoot elongation (Ma et al., 2010). Ethylene plays important roles in these adaptations (Saika et al., 2007; Steffens and Sauter, 2010; Ma et al., 2010; Steffens et al., 2012). Remarkably, in the dark, rice has a double response to ethylene (promoted coleoptile elongation and inhibited root growth) (Ma et al., 2010, 2013; Yang et al., 2015) that is different from the Arabidopsis triple response (short hypocotyl, short root, and exaggerated apical hook) (Bleecker and Kende, 2000). Several homologous genes of Arabidopsis ethylene signaling components have been identified in rice, such as the receptors, RTE1-like gene, EIN2-like gene, EIN3-like gene, CTR2, and ETHYLENE RESPONSE FACTOR (ERF) (Cao et al., 2003; Jun et al., 2004; Mao et al., 2006; Rzewuski and Sauter, 2008; Wuriyanghan et al., 2009; Zhang et al., 2012; Ma et al., 2013; Wang et al., 2013). We previously studied the kinase activity of rice ETR2 and the roles of ETR2 in flowering and in starch accumulation (Wuriyanghan et al., 2009). We also isolated a set of rice ethylene response mutants (mhz) and identified MHZ7/EIN2 as the central component of ethylene signaling in rice (Ma et al., 2013).

Here, we focused our studies on another ethylene-responsive mutant, mhz5, which, in the presence of ethylene, exhibits reduced sensitivity of root growth but enhanced sensitivity of coleoptile growth. Through map-based cloning, we found that MHZ5 encodes a carotenoid isomerase. Further physiological and genetic studies revealed that ethylene regulates carotenoid biosynthesis in rice and that the ethylene-induced inhibition of rice root growth requires the MHZ5/CRTISO-mediated ABA pathway. This latter feature is different from that in Arabidopsis, in which ethylene regulates root growth does not require ABA function. Additionally, a MHZ5/CRTISO mutation enhances ethylene production and EIN2-mediated coleoptile elongation. Our study provides important insight into the interactions of ethylene, carotenogenesis, and ABA in the regulation of rice growth and development.

RESULTS

Phenotype and Ethylene Response of Dark-Grown mhz5 Mutant Rice

Rice mhz5 is a previously described ethylene response mutant, and three mutant alleles of mhz5 (mhz5-1, mhz5-2, and mhz5-3) have been identified (Ma et al., 2013). Upon exposure to ethylene, root growth of wild-type etiolated rice seedlings was inhibited by ~80%, but coleoptile growth was promoted (Figure 1). By contrast, root growth of etiolated mhz5-1 seedlings was only partially inhibited (by ~35%) (Figures 1A, 1C, and 1D). Ethylene-induced coleoptile elongation was greater in mhz5-1 than that in the wild type (Figures 1A and 1B). The two allelic mutants mhz5-2 and mhz5-3 showed a similar ethylene response (Figures 1B to 1D). These results indicate that the mhz5 mutant has hypersensitivity in ethylene-promoted coleoptile elongation but reduced sensitivity in ethylene-inhibited root growth. In addition, three alleles of mhz5 display significantly (P < 0.01) shorter roots and slightly but significantly (P < 0.05) longer coleoptiles than those of the wild type in the absence of ethylene (Figures 1A to 1C). The three mhz5 alleles were phenotypically indistinguishable; therefore, two alleles, mhz5-1 and mhz5-3, were used for most of the analyses described below.

To further examine the ethylene response of the mhz5 mutant, we analyzed the transcript level of ethylene-responsive genes that were originally identified from a microarray assay (GSE51153). The expression of six genes, Photosystem II 10 kD
Figure 1. Phenotype and Ethylene Response of mhz5.

(A) Morphology of etiolated seedlings from 3-d-old wild-type and mhz5-1 seedlings in the presence of 10 ppm ethylene or air. Bars = 10 mm.

(B) Ethylene dose–response curves for the coleoptile length of 3-d-old dark-grown seedlings. The values are means ± SD of 20 to 30 seedlings per genotype at each dose.

(C) Ethylene dose–response curves for root length. The growth condition and statistical analyses are as in (B).

(D) Relative root length of the wild type and mhz5 mutants in response to ethylene (ethylene-treated versus untreated). Others are as in (B).
polypeptide, AP2 domain-containing protein (ERF063 and ERF073), cupin domain-containing protein (Gemin-like and RGLP1), and receptor-like kinase (SHRS), was upregulated by ethylene to varying degrees in the wild-type shoots as detected via quantitative realtime PCR (qRT-PCR). In mhz5-1 mutant shoots, the expression levels of these genes were higher than those in the wild type without ethylene treatment and were further enhanced by ethylene treatment (Figure 1E). Four other genes, including A-type response regulator (RRA5), B3 DNA binding domain-containing protein (RAP2.8), AP2 domain-containing protein (ERF002), and an auxin-responsive Aux/IAA family member (IAA20), were preferentially induced by ethylene in wild-type roots but not induced in mhz5-1 roots (Figure 1F). Shoots instead of coleoptiles were used for gene expression analysis because rice coleoptiles and shoots have a similar ethylene response (Ku et al., 1970). These results indicate that the mhz5-1 mutant is hypersensitive to ethylene in coleoptiles but less sensitive in roots in the expression of the ethylene-responsive genes.

Phenotypes of Field-Grown mhz5 Mutant Rice Plants

Adult field-grown mhz5 mutant plants had excessive tillers, smaller panicles, and fewer primary and secondary branches in panicles compared with wild-type plants (Supplemental Figure 1). The lengths of all internodes were shorter in mhz5-1 than the wild type (Supplemental Figure 1A). At the late tillering stage, the tiller numbers of mhz5 were drastically increased compared with the wild type (Supplemental Figures 1A and 1D). After harvest, the length and width of well-filled grains were measured, and all three allelic mutant grains were longer and narrower than those of the wild type. Consistently, the ratio of grain length/width was also apparently increased in mhz5 (Supplemental Figure 1E). In addition, the length of the primary roots, adventitious roots, and lateral roots of mhz5-1 seedlings were shorter than that of wild-type seedlings. Furthermore, mhz5-1 mutants had fewer adventitious roots but more lateral roots than the wild type (Supplemental Figure 2). These results indicate that MHZ5 disruption strongly affects agronomic traits.

Positional Cloning and Identification of MHZ5

We used a map-based cloning strategy to isolate the MHZ5 gene. The mhz5-1 mutant was crossed with four indica cultivars (93-11, MH63, ZF802, and TN1), and F2 populations were screened and mapped. A DNA sequence analysis of all 10 of the annotated genes within the mapped region revealed that the LOC_Os11g36440 had a single base pair substitution (A-T) in the eleventh exon at nucleotide 3114, and this mutation disrupted the splicing signal, resulting in a loss of 4 bp in cDNA, generating a premature translation termination product in mhz5-1 (Figure 2). Mutations in mhz5-2 and mhz5-3 were also identified in the same locus by sequencing and are indicated in Figures 2A to 2C. A single base pair substitution (G to C) in mhz5-2 at 313 bp caused a change of Gly-105 to Arg-105 (Figures 2A and 2B). In mhz5-3, a deletion of 26 bp from nucleotides 383 to 409 disrupted the splicing signal and resulted in aberrant splicing, causing the mRNA of mhz5-3 to be 475 bp longer than that in the wild type (Figures 2A to 2C). Although this mutation does not appreciably affect the mRNA level (Figure 2C, left panel), it leads to a truncated protein of 157 amino acids. The mhz5-1 and mhz5-2 mutations were confirmed via a derived cleaved amplified polymorphic sequence assay using PCR (Figure 2C, right panel), and the mhz5-3 mutation was confirmed via an amplified fragment length polymorphism assay using PCR (Figure 2C, right panel). A Tos17 retrotransposon insertion in the seventh exon of LOC_Os11g36440 (mh z5-4) (NG0489 from the rice Tos17 Insertion Mutant database, http://tos.nias.affrc.go.jp/~miyao/pub/tos17/index.html.en) completely disrupted the gene and generated an altered ethylene response that was similar to that in the mhz5-1 mutant (Figures 2A and 2B; Supplemental Figure 3).

The identity of mhz5 was confirmed by genetic complementation. Plasmid pMHZ5C, harboring a 7.13-kb genomic DNA fragment consisting of the 2278-bp upstream sequence, the entire MHZ5 gene, and an 1169-bp downstream region, was introduced into mhz5-3. Transgenic lines harboring the entire MHZ5 genomic sequence displayed the same ethylene response and phenotypes as those of wild-type plants (Figures 2D and 2E). These results confirm that MHZ5 is located at the locus LOC_Os11g36440, whose mutation leads to an alteration of the ethylene response and agronomic traits in rice.

Disruption of the Carotenoid Biosynthesis Pathway Mimics the Ethylene Response Phenotypes of the mhz5 Mutant

The MHZ5 gene encodes CRTISO, which catalyzes the conversion of 7,9,9′,7′-tetra-cis-lycopene (prolycopene) to all-trans-lycopene in the carotenoid biosynthesis pathway in plants (Isaacson et al., 2002; Park et al., 2002; Fang et al., 2008). We tested whether blocking the carotenoid biosynthesis pathway with an inhibitor of fluridone (Flu) at an early step of the conversion of phytoene to phytofluene (Hoffmann-Benning and Kende, 1992; Jamil et al., 2010) would similarly alter the ethylene response in wild-type rice seedlings. When Flu was added, the relative coleoptile length and the relative root length of wild-type seedlings significantly increased in the presence of ethylene (Figure 3), suggesting enhanced and reduced ethylene responses in coleoptiles and in roots, respectively. The Flu-treated wild-type seedlings resembled the mock mhz5-1 mutant when both were subjected to
Figure 2. Positional Cloning of the MHZ5 Gene.

(A) Fine mapping of the MHZ5 gene. The MHZ5 locus was mapped to the long arm of chromosome 11 between markers Idl11-20.3 and Idl11-21.2. Numerals under the markers indicate the number of recombinants identified from 589 F2 mutant plants. AC136149, AC108871, AC109929, and AC137589 are BAC clones. The location of MHZ5 was then fine mapped to a 152-kb genomic DNA region between markers Idl11-20.557 and Idl11-20.709. LOC_Os11g36440 is the candidate gene for MHZ5 and mhz5-4 represents a Tos17 insertion mutant (NG0489).

(B) The mutation sites of four allelic mutants of MHZ are shown superimposed on the structure of MHZ5 as predicted using SMART software (http://smart.embl-heidelberg.de). The black box represents the FAD-dependent oxidoreductase.

(C) Confirmation of mutation sites in mhz5-1, mhz5-2, and mhz5-3 via PCR-based analyses. The full-length cDNA of mhz5-1 and mhz5-2 was similar to the wild type, but that of mhz5-3 was 475 bp longer than that of the wild type (left panel). The PCR-amplified fragment from genomic DNA of mhz5-1 was 25 bp longer than that of the wild type digested with PvUII, the fragment from mhz5-2 was 27 bp shorter than that of the wild type digested with HhaI, and the fragment from mhz5-3 was 26 bp shorter than that of the wild type (right panel).

(D) Functional complementation of the mhz5-3 mutant. The complementation plasmid containing the entire MHZ5 (pMHZ5C) was transformed into mhz5-3 plants, rescuing the ethylene response phenotypes of mhz5-3 etiolated seedlings in transgenic lines (mhz5-3c) 6 and 14 (lower panel). The mhz5-3 mutant backgrounds in transgenic lines 6 and 14 were confirmed using PCR-based analyses with genomic DNA (upper panel). The fragment of mhz5-3 mutant was 26 bp shorter than the wild type. Bars = 10 mm.

(E) Functional complementation of the mhz5 mutant in the field. Methods are as in (D). Bar = 10 cm.
Figure 3. Disruption of the Carotenoid Biosynthesis Pathway Mimics the Ethylene Response Phenotypes of the mhz5 Mutant.

(A) Ethylene response phenotypes of 3-d-old dark-grown wild type and mhz5-1 mutants with or without a Flu inhibitor. The Flu-treated wild-type seedlings resembled the phenotypes of mhz5-1 in the presence of ethylene. Bars = 10 mm.

(B) Relative coleoptile length (ethylene-treated versus untreated in the wild type and mhz5-1, respectively) of the wild type and mhz5-1 that were treated with or without Flu in the presence or absence of ethylene. Values are means ± SD for 20 to 30 seedlings per genotype. A statistical analysis was performed using a one-way ANOVA (LSD t test) for ethylene-treated groups with statistical software (SPSS 18.0) (P < 0.05). Values for a and b are significantly different at P = 0.0008; values for b and c are significantly different at P = 0.005. Different letters above each column indicate significant difference between the compared pairs (P < 0.05).

(C) Relative root length of the wild type and mhz5-1. The seedlings treatment condition and statistical analyses are as in (B). Values for b and c are significantly different at P = 0.013.

(D) Ethylene response of 3-d-old light-grown wild-type, mhz5-1, and ein2 seedlings in ethylene or air. Bars = 10 mm.

(E) Relative root length (ethylene-treated versus untreated in the wild type and mutant, respectively) of 3-d-old light-grown rice seedlings at various concentrations of ethylene. Means ± SD are shown for 20 to 30 seedlings per genotype at each dose.

(F) and (G) Pigment analysis of the leaves of 4-d-old wild type and mhz5-1 mutants that were either etiolated (F) or exposed to light for 24 h (G). N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Ca, chlorophyll a; Cb, chlorophyll b; pLy, protoporphyrin; Ne, neurosporene; Z, zeaxanthin; tLy all-trans-lycopene; β, β-carotene. Absorbance was at 440 nm. mAU, milliabsorbance units. Each experiment was repeated at least three times with similar results.
ethylene treatment (Figures 3A to 3C), demonstrating that the impairment of the carotenoid biosynthetic pathway affects ethylene responses in rice seedlings.

Light treatment can convert prolycopene to all-trans-lycopene through photoisomerization, partially replacing the functions of carotenoid isomerase (Isaacson et al., 2002; Park et al., 2002). We investigated whether light would affect the ethylene response of mhz5-1 compared with the wild type and the ethylene-insensitive mutant ein2/mhz7-1 (Ma et al., 2013). Upon exposure to continuous light, the roots of the mhz5-1 mutant had the same ethylene response as the wild type at different concentrations of ethylene. By contrast, the mutant ein2/mhz7-1 was still insensitive to ethylene in roots in the light (Figures 3D and 3E). These results indicate that light can rescue the ethylene response phenotype of mhz5-1 roots, indicating that carotenogenesis mediates the regulation of ethylene responses in rice seedlings.

To elucidate the mechanisms of the different ethylene responses of mhz5-1 in the dark and light, we analyzed the carotenoid profiles of the leaves and roots of wild-type and mhz5-1 seedlings. Unlike the profile of wild-type etiolated leaves, the mhz5-1 etiolated leaves accumulated prolycopene, the substrate of MHZ5/carotenoid isomerase for the conversion to all-trans-lycopene (Figure 3F). Neosperone, a substrate for ω-carotene desaturase that is immediately upstream of the MHZ5 step, was also accumulated in the mhz5-1 etiolated leaves (Figure 3F). In the mhz5-1 roots, only prolycopene was detected (Supplemental Figure 4). These results indicate that MHZ5 mutation leads to the accumulation of prolycopene, the precursor of all-trans-lycopene in the leaves and roots of mhz5-1 seedlings.

Upon exposure to light, there was a rapid decrease in the prolycopene level in mhz5-1 leaves and roots (Figures 3F and 3G; Supplemental Figures 4A and 4B). Furthermore, increases in the contents of all-trans-lycopene, zeaxanthin, and antheraxanthin were apparently observed in light-treated mhz5-1 leaves compared with those in wild-type leaves (Figure 3G). Levels of other carotenoids and the photosynthetic pigments were comparable between the mhz5-1 and wild-type leaves, except for the lower level of lutein in mhz5-1 compared with that of the wild type (Figure 3G, Table 1). In the roots of light-treated mhz5-1, prolycopene has been converted to the downstream metabolites, and the content of neoaxanthin was very similar to that in the wild type (Supplemental Figure 4B). These results suggest that light treatment leads to the conversion of prolycopene to all-trans-lycopene and to the further biosynthesis of downstream metabolites, rescuing the mhz5-1 ethylene response.

In the dark, the accumulation of prolycopene leads to an orange-yellow coloration in the mhz5-1 leaves, different from the yellow leaves of the wild-type seedlings. Additionally, the mhz5-1 seedlings had a markedly delayed greening process when exposed to light (Supplemental Figure 5), most likely due to the low efficiency of photoisomerization and/or the abnormal development of chloroplasts (Park et al., 2002).

Fluor inhibitor tests and light rescue experiments indicate that the aberrant ethylene response of mhz5-1 may result from the lack of carotenoid-derived signaling molecules. Considering that field-grown mhz5-1 plants have more tillers than do wild-type plants (Supplemental Figure 1), and carotenoid-derived SL inhibits tiller development (Umehara et al., 2008), we examined whether SL is involved in the aberrant ethylene response of the mhz5-1 mutant. We first analyzed 2′-epi-5-deoxyxtrigol (epi-5DS), one compound of the SLs in the exudates of rice roots and found that the concentration of epi-5DS in mhz5-1 was lower than that in the wild type (Supplemental Figure 6). We then tested the effect of the SL analog GR24 on the ethylene response and found that GR24 could not rescue the ethylene response of the mhz5-1 mutant (Supplemental Figures 6B and 6C). Additionally, inhibiting the SL synthesis gene D17 encoding the carotenoid cleavage dioxygenase (Zou et al., 2006) or the SL signaling gene D3 encoding an F-box protein with leucine-rich repeats (Zhao et al., 2014) in transgenic rice did not alter the ethylene response, although these transgenic plants had more tillers, a typical phenotype of a plant lacking SL synthesis or signaling (Supplemental Figures 6D and 6E). These results suggest that the abnormal ethylene responses of mhz5-1 etiolated seedlings do not appear to be consequences of altered SL synthesis or signaling.

**Ethylene Inhibition of Etiolated Rice Seedling Root Growth Requires the MHZ5-Mediated ABA Biosynthesis**

ABA is another important signaling molecule that is derived from the carotenoid biosynthesis pathway (Nambara and Marion-Poll, 2005). We measured the ABA contents in wild-type and mhz5-1 mutant etiolated seedlings and found that the mhz5-1 mutant had very low levels of ABA compared with the wild type (Figure 4), indicating that MHZ5/CRTISO is essential for ABA biosynthesis in etiolated shoots and roots. Because mhz5-1 has very little ABA, we examined whether the addition of ABA could complement the phenotypes of the mhz5-1 mutant. Without ethylene treatment, the application of 0.04 μM ABA restored the short roots of the mhz5 mutant to the wild-type level under normal conditions (Figure 4B), suggesting that basal levels of endogenous ABA are essential for the maintenance of root growth. We further tested whether ABA could restore the ethylene response of mhz5-1. In the presence of 10 ppm ethylene, the application of 0.1 μM ABA could largely rescue the ethylene sensitivity of mhz5-1 coleoptiles and roots (Figures 4C to 4E). This ABA concentration (0.1 μM) had no effect or only a slightly inhibitory effect on coleoptile and root growth in wild-type etiolated seedlings (Supplemental Figure 7). These results suggest that

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak Area Ratio for mhz5-1/Wild Type</th>
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<tbody>
<tr>
<td>Neoxanthin</td>
<td>0.94 ± 0.01**</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>1.26 ± 0.09**</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.18 ± 0.004**</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.75 ± 0.02**</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.91 ± 0.01*</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>1.22 ± 0.08**</td>
</tr>
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Values are means ± s.d. of three biological replicates. Student’s t test (**P < 0.01; *P < 0.05).
Figure 4. Ethylene Inhibition of Etiolated Rice Seedling Root Growth Requires the MHZ5-Mediated ABA Pathway.

(A) Influence of ethylene on ABA accumulation in the shoots and roots of wild-type and mhz5-1 mutant seedlings. Three-day-old etiolated seedlings were treated with or without ethylene (10 ppm) for 24 h. The values are the means ± so from three biological replicates. Asterisks represent significant difference between ethylene-treated and untreated in wild-type seedlings.

(B) The root defect of mhz5-1 is rescued by ABA. Wild-type and mhz5-1 seedlings were grown in the dark in solutions with or without 0.04 μM ABA for 2.5 d. Values are means ± so of 30 seedlings per genotype.

(C) ABA rescues the ethylene response of mhz5-1. The wild type and mhz5-1 were incubated in solutions with or without 0.1 μM ABA and treated with or without 10 ppm ethylene for 2.5 d. The coleoptiles of the wild type and mhz5-1 were sprayed once daily with 0.1 μM ABA (containing 0.001% Tween 20) after germination. The mock solution contains 0.1% ethanol and 0.001% Tween 20. Bars = 10 mm.

(D) Absolute coleoptile length of 2.5-d-old dark-grown wild-type and mhz5-1 seedlings that were incubated in solutions with or without 0.1 μM ABA and treated with or without ethylene. Values are means ± so of 20 to 30 seedlings per genotype. Asterisks represent significant difference between mhz5 with ABA, and mhz5 without ABA under ethylene-treated conditions.

(E) Relative root length (ethylene-treated versus untreated in the wild type and mhz5-1, respectively). Others are as in (D). Asterisks represent significant difference between mhz5 with ABA and mhz5 without ABA under ethylene-treated conditions.

(F) MHZ5 was induced in wild-type roots by ethylene as detected using qRT-PCR. RNA was isolated from 3-d-old wild-type etiolated seedlings after treatment with 10 ppm ethylene, 1-MCP (an ethylene competitive inhibitor), or air for various times. The values are the means ± so of three biological replicates.

(G) MHZ5 was induced in wild-type shoots by ethylene. The seedlings growth treatment and qRT-PCR strategies are as in (F).
ethylene requires ABA function to regulate the ethylene response in etiolated rice seedlings.

We then examined the effects of ethylene on ABA concentration and found that ethylene inhibited ABA accumulation in wild-type shoots but promoted ABA accumulation in wild-type roots, suggesting the tissue-specific regulation of ABA accumulation (Figure 4A). We further investigated the MHZ5 transcript with ethylene treatment and found that this transcript was induced by ethylene in both the roots and shoots (Figures 4F and 4G). These results indicate that ethylene promotes ABA accumulation in wild-type roots, possibly, in part, through the induction of MHZ5 expression. In the wild-type shoots, the discrepancy between ethylene-inhibited ABA accumulation and ethylene-induced MHZ5 expression is most likely due to ethylene-activated ABA catabolism for homeostasis in the shoots (Benschop et al., 2005; Saika et al., 2007).

Because ethylene induced the accumulation of ABA in wild-type roots, we further tested whether the carotenoid profile was altered by ethylene treatment. The contents of neoxanthin, the substrate of the rate-limiting enzyme NCED in the ABA biosynthetic pathway, increased by 42% (P = 0.0024) in the wild type with ethylene treatment (Figures 4H and 4I). By contrast, neoxanthin was not detected in mhz5-1 roots either with or without ethylene due to the disruption of the carotenoid biosynthetic pathway.

To further investigate the role of ethylene-triggered ABA in the rice root ethylene response, we measured the effects of ABA biosynthesis inhibitor nordihydroguaiaretic acid (NDGA) on ethylene-induced ABA accumulation as well as ethylene-inducible gene expression. NDGA inhibits the enzyme NCED in the ABA biosynthesis pathway (Creelman et al., 1992; Zhu et al., 2009). In the presence of NDGA, the ABA accumulation in the roots was nearly 30% that in untreated wild type, and ethylene-triggered ABA accumulation was completely blocked in the roots (Figure 4J). IAA20 can be induced by ethylene in the wild-type roots but not in the mhz5-1 roots (Figure 1F). This gene can also be induced by ABA in wild-type roots (Figure 4K). However, the ethylene induction of IAA20 was almost completely abolished in the presence of NDGA (Figure 4K), suggesting that ethylene-induced gene expression requires ABA function. In summary, the above results suggest that the ethylene inhibition of rice root growth requires MHZ5-mediated ABA biosynthesis.

**mhz5 Etiolated Seedlings Produce More Ethylene, and Their Coleoptile Response to Ethylene Mainly Results from Enhanced Ethylene Signaling**

As shown in Figures 4C and 4D, the enhanced ethylene response in mhz5-1 coleoptiles was substantially inhibited by ABA, suggesting that ABA deficiency is the major reason for the hypersensitivity of mhz5-1 coleoptiles to ethylene. An enhanced ethylene response could result from ethylene overproduction and/or enhanced signal transduction. Thus, we examined whether ethylene production is altered in mhz5-1. As shown in Figure 5, mhz5-1 etiolated seedlings produced nearly 3 times as much ethylene than did the wild type (based on fresh weight), and ABA addition dramatically suppressed ethylene production in the mhz5-1 mutant. These results indicate that MHZ5-mediated ABA biosynthesis inhibits ethylene production in etiolated rice seedlings. It should be noted that ethylene production in light-grown seedlings is very similar to that in the wild type, further demonstrating that light could substitute for MHZ5 isomerase activity through photoisomerization as previously described (Isaacs on et al., 2002; Park et al., 2002). We further studied the expression of ethylene biosynthesis genes and found that the ACS2, ACS6, and ACS5 levels were all higher in both the shoots and roots of the mhz5-1 etiolated seedlings than those in the wild-type seedlings (Figure 5B). Notably, the ACS3 level was higher in the shoots of mhz5-1 than that in the wild-type shoots. However, expression of this gene was very similar in the roots of the wild type and mhz5-1 mutant (Figure 5B). The differential expression of ACS3 most likely reflects tissue-specific and/or posttranscriptional regulation. These results suggest that enhanced ethylene emission in mhz5-1 plants is likely due to the increased expression of ethylene biosynthesis-related genes.

mhz5-1 had slightly but significantly (P < 0.05) longer coleoptiles than did the wild type in the dark in the absence of ethylene treatment (Figures 5C and 5D). L-α-(2-aminoethoxyvinyl)-glycine (AVG), the ethylene biosynthesis inhibitor, can effectively block the ethylene production of the mhz5-1 mutant and wild type (Supplemental Figure 8). When AVG was included, the basal elongation of the mhz5-1 coleoptiles was reduced to the level of the wild type without AVG treatment (Figures 5C and 5D; Supplemental Figure 8B). These results indicate that endogenously overproduced ethylene contributes to the basal coleoptile elongation of the
mhz5-1 mutant. However, in the presence of AVG, when a wide range of exogenous ethylene was applied, the coleoptile elongations of mhz5-1 were still greater than those of the wild type (Figures 5C and 5D). These results suggest that the endogenous ethylene production of mhz5-1 does not contribute to the hypersensitive response of mhz5-1 coleoptiles to ethylene.

We further found that the transcript level of EIN2 was higher in mhz5-1 shoots than that in the wild type in the absence of ethylene. By contrast, this transcript was not upregulated in the roots of the mhz5-1 mutant (Figure 5E). Taken together, these data suggest that the enhanced ethylene response of mhz5-1 coleoptiles most likely results from enhanced ethylene signaling due to higher EIN2 expression.

MHZ5 Overexpression Alters the Ethylene Response in Rice

To further elucidate the function of MHZ5, the MHZ5-coding sequence driven by the cauliflower mosaic virus 35S promoter was introduced into wild-type plants, and four homozygous independent MHZ5-overexpression lines (MHZ5-OE) were used for analysis (Figure 6). The four dark-grown transgenic lines all displayed slightly but significantly shorter coleoptiles (P < 10^-6) and roots (P < 10^-6) compared with those of the wild type in air (Figures 6B to 6D). When treated with exogenous ethylene, the coleoptile elongation of MHZ5-OE lines was less than that in the wild type (Figures 6B and 6C), suggesting the presence of reduced coleoptile ethylene sensitivity. However, the inhibition of

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**Figure 5.** The mhz5 Etiolated Seedlings Produce More Ethylene Than the Wild Type, and Their Elevated Coleoptile Response to Ethylene Mainly Results from Enhanced Ethylene Signaling.

(A) Ethylene production of etiolated seedlings and green seedlings. The values are the means ± SD from four biological replicates. Asterisks indicate a significant difference between the wild type without ABA treatment and mhz5-1 etiolated seedlings at P < 0.01 using Student’s t test.

(B) Relative expression level of ethylene synthesis genes in wild-type and mhz5-1 mutant seedlings. RNA was extracted from the shoots and roots of 3-d-old etiolated seedlings and used for qRT-PCR. Values are means ± SD from three biological replicates.

(C) The effect of the ethylene biosynthesis inhibitor AVG (50 μM) on the ethylene response of wild-type and mhz5-1 seedlings. Seedlings were incubated on eight layers of cheesecloth in Petri dishes in a plastic box with or without 10 ppm ethylene for 2.5 d. Bars = 10 mm.

(D) Coleoptile length of wild-type and mhz5-1 seedlings in response to ethylene following the addition of 50 μM AVG. Values are mean lengths ± SD of 20 to 30 seedlings.

(E) EIN2 transcript levels in the shoots and roots of 3-d-old etiolated wild-type and mhz5-1 seedlings as detected using RT-PCR. Actin1 was used as the loading control. Each experiment was repeated at least three times with similar results.
root growth of the MHZ5-OE lines was more severe than that in the wild type, especially under 1 ppm ethylene treatment (Figures 6B, 6D, and 6E), suggesting enhanced root ethylene sensitivity. The roots of the MHZ5-OE lines were all shorter than those of the wild type under normal conditions, and this short-root phenotype is similar to that of the mhz5-1 mutants (Figures 1C and 6D). The short-root phenotype in these plants most likely resulted from altered ABA levels because a normal level of ABA is essential for root growth in plants (Yin et al., 2009; Zhang et al., 2010; Wang et al., 2011). MHZ5 expression levels seemed to roughly correlate with the ethylene response in the coleoptiles and roots of the transgenic plants (Figures 6A to 6E).

To further establish the ethylene responsiveness of MHZ5-OE, we examined the expression of ethylene-inducible genes using qRT-PCR. Transcript levels of ethylene-inducible genes were comparable in the wild-type and MHZ5-OE lines in the air (Figures 6F and 6G). Upon exposure to ethylene, ethylene induction of Germin-like and SHR5 was significantly lower in the MHZ5-OE shoots than those in the wild-type shoots (Figure 6F). In the roots, the induced levels of RRA5 and ERF002 were significantly higher in the MHZ5-OE lines than those in the wild type (Figure 6G). These results indicate that the overexpression of MHZ5 reduced the expression of a subset of ethylene-responsive genes in coleoptiles but promoted the expression of another subset of ethylene-responsive genes in the roots of etiolated seedlings. Furthermore, in the shoots/coleoptiles, the transcript level of EIN2 was lower to varying degrees in the MHZ5-OE lines than that in the wild type (Figure 6H), suggesting that the reduced ethylene responsiveness of the shoots/coleoptiles most likely results from the reduction of ethylene signaling. These gene expression patterns in MHZ5-OE plants are consistent with those in mhz5-1 mutant (Figures 1E, 1F, and 5E). Together, these results indicate that MHZ5 differentially affects the ethylene response of rice shoots/coleoptiles and roots at the gene expression level.

**Genetic Interactions of MHZ5 with Ethylene Signaling Components in Rice**

To examine the genetic interactions of MHZ5 with ethylene receptor genes, double mutants were generated between mhz5-1 and three ethylene receptor mutants. The three receptor single loss-of-function rice mutants ers1, ers2, and etr2 were in the background of the japonica variety Dongjin (DJ), and their T-DNA insertions in the corresponding genes were identified using PCR-based genotyping (Supplemental Figure 9). The three ethylene receptor mutants showed no significant change in coleoptile length. However, their roots were significantly shorter than the wild type and displayed a moderately enhanced ethylene response compared with that in the background variety DJ. The root ethylene responses of the three double mutants (ers1 mhz5-1, ers2 mhz5-1, and etr2 mhz5-1) were very similar to that of mhz5-1 alone (Figure 7). These results indicate that the ethylene receptor single mutants require an MHZ5-mediated pathway to display the ethylene response phenotype in the roots or that the MHZ5-mediated pathway acts downstream of the three ethylene receptors ERS1, ERS2, and ETR2 to regulate the root ethylene response.

A double mutant was also produced by crossing homozygous mhz5-3 with ein2. ein2/mhz7-1 was identified as an ethylene-insensitive mutant in our previous study (Ma et al., 2013). In etiolated seedlings, ein2 completely suppressed the coleoptile elongation phenotype of mhz5-3 in a wide range of ethylene concentrations (Figure 8), indicating that the coleoptile ethylene response of mhz5 requires EIN2 signaling. The roots of the mhz5-3 ein2 double mutant displayed an absolute insensitivity to each concentration of exogenous ethylene (Figures 8A and 8C), suggesting that EIN2 and MHZ5 most likely act within the same pathway for ethylene-induced root inhibition.

To further examine the genetic relationship between MHZ5 and the ethylene signaling component EIN2, we analyzed the ethylene response of the mhz5-3 EIN2-OE3 plants that were obtained by crossing homozygous mhz5-3 with EIN2-OE3 (EIN2-overexpression transgenic line; Ma et al., 2013). The coleoptiles of mhz5-3 EIN2-OE3 homozygous plants were more elongated than the mhz5-3 and EIN2-OE3 seedlings that were treated with or without 1 ppm ethylene (Figures 8D and 8F). By contrast, the root growth of mhz5-3 EIN2-OE3 homozygous plants displayed a twisted phenotype in the seminal root in the air compared with that of EIN2-OE3 seedlings (Figures 8D and 8E). This phenotype was most likely due to ABA deficiency and/or ethylene overproduction. Upon exposure to ethylene, the inhibition of root growth of EIN2-OE3 seedlings was partially alleviated in the mhz5-3 EIN2-OE3 seedlings; however, the waved/twisted root phenotype remained similar or was more severe in the mhz5-3 EIN2-OE3 seedlings that were treated with ethylene compared with the seedlings without ethylene treatment (Figures 8D, 8E, 8G, and 8H). These data suggest that the MHZ5-mediated pathway is partially required by EIN2 signaling for the regulation of the ethylene-induced inhibition of root growth.

We further generated ein2 MHZ5-OE48 plants by crossing the ein2 mutant with MHZ5-OE48 plants overexpressing the MHZ5 gene (Figure 6). The coleoptiles of the double mutant seedlings were like those of ein2 with or without ethylene (Figures 8I and 8J). However, with the ethylene treatment, the relative root length was mildly but significantly reduced in the ein2 MHZ5-OE48 seedlings compared with that in ein2 (Figures 8I and 8K). These results indicate that MHZ5 can partially suppress root ethylene insensitivity in the ein2 mutant.

**DISCUSSION**

In this study, we characterized the rice ethylene response mutant mhz5, which displays an enhanced ethylene response in coleoptile elongation but a reduced ethylene response in root inhibition. We determined that MHZ5 encodes a carotenoid isomerase in the carotenoid biosynthesis pathway, facilitating metabolic flux into the biosynthesis of ABA precursors and ABA. Ethylene induces MHZ5 expression and accumulation of the ABA biosynthesis precursor neoxanthin and ABA in roots. ABA largely rescues the ethylene response in both the coleoptiles and roots of mhz5 etiolated seedlings. Genetically, the MHZ5-mediated ABA pathway acts downstream of ethylene signaling to regulate root growth in rice. This interaction between ethylene and ABA is different from that in Arabidopsis, where ABA...
Figure 6. MHZ5 Overexpression Alters the Ethylene Response in Rice.

(A) MHZ5 expression levels in shoots and roots of 3-d-old dark-grown wild type and four MHZ5 overexpression lines. Values are the means ± sd of three biological replicates.

(B) Phenotypes of the wild type and various MHZ5 overexpression lines in response to ethylene. The 2.5-d-old dark-grown seedlings of the wild type and four independent transgenic lines were treated with or without 1 ppm ethylene. Bar = 10 mm.

(C) Effect of ethylene on coleoptile length. Values are means ± sd of 20 to 30 seedlings per genotype.

(D) Effect of ethylene on root length. Values are means ± sd of 20 to 30 seedlings per genotype.

(E) Relative root length of wild-type and transgenic lines in response to ethylene (ethylene-treated versus untreated in the wild type and MHZ5-OE lines, respectively). The data are derived from (D).

(F) Expression of ethylene-responsive genes in the shoots of the wild type and four transgenic lines. Three-day-old dark-grown seedlings were treated with or without ethylene (10 ppm) for 8 h, and total RNA was extracted for qRT-PCR. Values are means ± sd of three biological replicates.

(G) Expression levels of genes preferentially induced by ethylene in the roots. Others are as in (F).

(H) EIN2 transcript levels in the shoots of 3-d-old etiolated seedlings of wild-type and MHZ5-OE lines as detected using RT-PCR. Actin1 served as the loading control. Each experiment was repeated at least three times with similar results.
requires ethylene signaling for root inhibition. By contrast, the MHZ5-mediated ABA pathway negatively regulates EIN2 signaling to control coleoptile growth. Our results reveal novel interplays among ethylene, carotenoid, and ABA in the regulation of the ethylene response in rice.

**An MHZ5-Mediated ABA Pathway Acts Downstream of Ethylene Signaling for Root Growth Inhibition in Etiolated Rice Seedlings**

We provide several lines of evidence to demonstrate that the MHZ5-mediated ABA pathway is required for the ethylene inhibition of root growth in rice. First, light treatment rescues the mhz5-1 root ethylene response through the photoisomerization of prolycopene into downstream metabolites. Second, blocking the carotenoid pathway with an inhibitor (Flu) led to aberrant ethylene response phenotypes in the wild type that are similar to the ethylene response in mhz5. Third, the exogenous application of ABA significantly recovers the mutant ethylene response. Fourth, ethylene induces MHZ5 expression, ABA biosynthesis precursor neoxanthin and ABA accumulation in wild-type roots, and ethylene-induced ABA accumulation depends on MHZ5 function. Fifth, ethylene-induced ABA mediates the expression of some ethylene-responsive genes. Sixth, MHZ5 overexpression leads to an enhanced ethylene response and promotes ethylene-induced gene expression in the roots. Seventh, genetic analysis suggests that ethylene signaling acts upstream of the MHZ5-mediated ABA pathway to regulate root growth (Figures 7 and 8). Additionally, other ABA-deficient mutants, such as mhz4/aba4 (Ma et al., 2014), aba1, and aba2, also

![Image of rice seedlings](image-url)
Figure 8. Genetic Interaction between MHZ5 and EIN2 in the Regulation of the Ethylene Response.

(A) Phenotypes of 3-d-old dark-grown seedlings in the presence or absence of ethylene (10 ppm). Bars = 10 mm.
exhibit reduced ethylene sensitivity in roots (Supplemental Figure 10). Moreover, higher concentrations of ABA inhibit root growth in etiolated rice seedlings (Supplemental Figure 7). From the above evidence, we propose that ethylene may exert its effects on root inhibition at least partially through the MHZ5-mediated ABA pathway (Figure 9).

Our finding that the ethylene inhibition of root growth in rice is at least partially ABA dependent is in contrast with that obtained in Arabidopsis, in which the ethylene inhibition of root growth is ABA independent, and ABA requires ethylene biosynthesis and signaling for root growth regulation (Beaudoin et al., 2000; Ghassemian et al., 2000; Cheng et al., 2009; Luo et al., 2014). This difference is mostly likely due to the different plant species that were used. The different living conditions of their seedlings, namely, the hypoxic environment in rice versus normal aerated soil in Arabidopsis, may also be the reason for this result. It is not clear whether other monocotyledonous seedlings have a similar mechanism.

The mhz5 mutant exhibits reduced sensitivity, but not complete insensitivity, to ethylene in root tips, and ethylene is still able to cause ~35% reduction in mhz5 root growth (Figure 1). These data suggest that ethylene can inhibit root growth through both an ABA-dependent and ABA-independent manner. Because the remaining ethylene response of the mhz5 roots was completely blocked by ein2, whose loss of function makes etiolated rice seedlings completely insensitive to ethylene (Ma et al., 2013), the ABA-independent ethylene response may rely on EIN2 and/or its downstream event. Taken together, these results demonstrate that the maximum inhibition of root growth by ethylene involves both ABA-dependent and ABA-independent functions and that the MHZ5-mediated ABA pathway may work together with the EIN2 downstream signaling pathway to coregulate the ethylene inhibition of root growth (Figure 9A).

The Role of MHZ5 in the Ethylene Regulation of Rice Coleoptile Elongation

Rice seedlings have a coleoptile for protection of emerging leaves. This feature is different from Arabidopsis seedlings. Ethylene promotes coleoptile elongation (Figure 1). ABA accumulation is reduced in the mhz5 mutant, whereas ethylene production is enhanced (Figures 5 and 6). The coleoptile elongation of mhz5 is promoted in response to ethylene (Figure 1), indicating a hypersensitive response in etiolated rice seedlings compared with that in the wild type. The enhanced ethylene response is mostly likely due to the high expression of EIN2 in mhz5 shoots and not due to the ethylene overproduction because the treatment with ethylene biosynthesis inhibitor AVG did not significantly affect the ethylene response of mhz5 (Figure 5). In addition, the hypersensitive ethylene response of mhz5 is fully dependent on EIN2 signaling through double mutant analysis (Figures 8A and 8B). These findings led us to conclude that the MHZ5-mediated ABA pathway inhibits ethylene production and negatively modulates ethylene signaling to control coleoptile elongation (Figure 9B). In a feedback control manner, ethylene could decrease ABA accumulation in the shoots coleoptile in terms of the coleoptile ethylene response, the possibility cannot be excluded that other ABA-deficient mutants of aba1 and aba2 (Supplemental Figure 10) were weaker than that of mhz5 in terms of the coleoptile ethylene response, the possibility cannot be excluded that other carotenoid-derived molecules (e.g., SL, BYPASS, and/or uncharacterized compounds) and/or interactions among different plant growth regulators may also contribute to regulation of coleoptile ethylene responses in rice.

In etiolated rice seedlings, crosstalk may occur at multiple levels between ethylene and ABA, such as the biosynthesis pathway, signaling pathway, or even responsive genes. Ethylene...
biosynthesis genes, such as ACS and ACO, were upregulated, and ethylene production increased significantly in mhz5 etiolated seedlings, suggesting that ethylene and ABA can act antagonistically at the biosynthesis level. These observations are consistent with those in the tomato mutant flacca (Tal, 1979) and the Arabidopsis mutants aba1 and aba2 (Rakitina et al., 1994; LeNoble et al., 2004). The data mentioned above suggest that the ABA inhibition of ethylene biosynthesis is conserved.

Ethylene Regulates Carotenoid Biosynthesis in Etiolated Rice Seedlings

Carotenoids are essential pigments that play pivotal roles in photoprotection (Niyogi, 1999; Dall’Osto et al., 2007; Wei et al., 2010; Ballottari et al., 2014). Carotenoid-derived compounds, such as SL, ABA, BYPASS, β-cyclocitral, and other uncharacterized molecules, modulate plant developmental processes and stress responses in many organs (Xie et al., 2010; Sieburth and Lee, 2010; Walter et al., 2010; Cazzonelli and Pogson, 2010; Puig et al., 2012; Ramel et al., 2012; Avendaño-Vázquez et al., 2014; Van Norman et al., 2014; Liu et al., 2015).

The regulation of carotenoid biosynthesis is interconnected with plant developmental and environmental responses, and the biosynthesis pathway is regulated at both the transcriptional and posttranscriptional levels in plants (Ruiz-Sola and Rodríguez-Concepción, 2012). Previous studies have found that the interaction between carotenogenesis and ethylene is mainly associated with tomato (Solanum lycopersicum) fruit ripening, in which ethylene influences multiple steps in carotenoid synthesis, impacting the net and relative accumulation of the compounds (Bramley, 2002; Alba et al., 2005). In this study, the ethylene-induced expression of the carotenoid isomerase gene MHZ5 drove the metabolic flux into the formation of ABA biosynthesis precursors, including neoxanthin, leading to ABA accumulation in the roots and to the root growth inhibition of etiolated rice seedlings (Figure 4). This conclusion is further supported by our recent finding that ethylene also induces the expression of rice ABA4 (Ma et al., 2014), a gene homologous to Arabidopsis ABA4, encoding a membrane protein that might regulate the conversion of zeaxanthin to neoxanthin in the ABA biosynthesis pathway (North et al., 2007). Additionally, ethylene induces the transcription of NCED in the ABA biosynthesis pathway and then the accumulation of ABA to modulate fruit ripening in grape berry (Vitis vinifera; Sun et al., 2010). These analyses suggest that ethylene regulates the carotenoid biosynthesis pathway at both the early steps, e.g., the conversion of prolycopene to all-trans-lycopene by carotenoid isomerase MHZ5 and the late steps in the ABA biosynthesis pathway to modulate rice seedling growth and/or the fruit ripening process.

Root tissue is a major site of ABA biosynthesis, where the low concentrations of carotenoid precursors may prove rate-limiting. Although only trace levels of neoxanthin and violaxanthin have been identified in the root tissue of plants (Parry and Horgan, 1992), the trace levels of carotenoids that are induced by ethylene play an important role in ABA biosynthesis to synergistically inhibit the root growth of etiolated rice seedlings (Figure 4). Additionally, in plant roots, the carotenoid biosynthesis rate-limiting enzyme PSY isogenes that are involved in the production of root carotenoids are induced by abiotic stress and specifically by ABA (Welsch et al., 2008; Meier et al., 2011; Ruiz-Sola and Rodríguez-Concepción, 2012). These findings indicate that carotenoid biosynthesis in the leucoplasts of roots is elaborately regulated by external and internal cues. It is possible that multiple regulation manners allow plants to be more adapted to the complicated and changing environment at different growth and developmental stages.

Shifting mhz5 seedlings from dark to light altered the carotenoid profile to the immediate precursors of ABA biosynthesis (Figure 3G), which is similar to those reported for light-grown seedlings of zebra2/crtiso, an allelic mutant of mhz5, when exposed to higher light intensities (Chai et al., 2011). However, our study reveals roles for the carotenoid isomerase/MHZ5 in regulation of ethylene responses. Additionally, the mhz5 mutant has complicated phenotypes in the field (Supplemental Figures 1 and 2) that have not been previously reported (Chai et al., 2011).
Field-grown mhz5 plants under environmental light conditions did not resemble wild-type plants, suggesting that light can only partially substitute for MHZ5/CRTISO activity, which is consistent with previous reports in Arabidopsis and tomato (Isaacson et al., 2002; Park et al., 2002). In addition to the current roles of the carotenoid-derived ABA pathway in the regulation of rice seedling growth, other carotenoid-derived molecules, e.g., SL, BYPASS, and uncharacterized compounds, may be responsible for tiller formation (Supplemental Figure 1), root development (Supplemental Figure 2), and other phenotypic changes in field-grown mhz5 plants (Nambara and Marion-Poll, 2005; Umehara et al., 2008; Sieburth and Lee, 2010; Kapulnik et al., 2011; Puig et al., 2012; Ramel et al., 2012; Van Norman et al., 2014).

In conclusion, we demonstrate that the carotenoid biosynthesis of rice is regulated by ethylene. Ethylene requires the MHZ5/carotenoid isomerase-mediated ABA pathway to inhibit root growth, and the MHZ5/carotenoid isomerase-mediated ABA pathway negatively regulates coleoptile elongation at least with previous reports in Arabidopsis and tomato (Isaacson et al., 2012; Ramel et al., 2012; Van Norman et al., 2014). In Arabidopsis, the manipulation of the corresponding components may improve agronomic traits and adaptive growth in rice.

**METHODS**

**Plant Materials and Growth Conditions**

mhz5, ein2/mhz7-1, and EIN2-OE3 were previously identified (Ma et al., 2013). The mhz5 allele mhz5-4 was obtained from Tos17 retrotransposon insertion lines (line numbers NG0489). The rice (Oryza sativa) aba1 and aba2 mutants were kindly provided by Cheng-Cai Chu (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The T-DNA knockout mutants ers1, ers2, and etr2 are in the DJ background and were obtained from the POSTECH Biotech Center (Yi and An, 2013). The primers that were used to identify homogenous ers1, ers2, and etr2 are listed in Supplemental Table 1. The ethylene treatments were performed as previously described (Ma et al., 2013) with the following modifications: 1.2 g of fresh weight tissue, 800 μL of acetone/ethylene acetate, and 620 μL of water during the sample extraction process. Due to the low level of carotenoids, pigment extraction and analysis in roots were performed as previously described (Fraser et al., 2000; Park et al., 2002). The relative abundance of each carotenoid was obtained by comparing the ratio of each peak area (the peak area of the synthesized compound divided by the peak area of the synthesized compound) to the total peak area of all compounds. For carotenoid assays, etiolated wild-type and mhz5-1 seedlings were grown in the dark for 3 to 4 d or the etiolated seedlings were treated with 10 ppm ethylene or transferred to continuous light for 24 h, after which the leaves and roots were frozen in liquid nitrogen for extractions.

**Vector Construction and Rice Transformation**

The complementation vector was constructed as follows. First, part of the MHZ5 genomic DNA fragment (containing the 2278-bp upstream sequence and a 1657-bp part of the coding region) was PCR amplified and ligated to a pCAMBIA2300 vector (provided by Cheng-Cai Chu) that was digested with XbaI and Sall to generate pMHZ5CM. The second part of the MHZ5 genomic DNA fragment (containing the 2018-bp left part of the coding region and the 1169-bp downstream region) was PCR amplified and ligated to the Sall and Sse8387 sites of the pMHZ5CM vector to form pMHZ5SC. To construct the MHZ5 overexpression vector, the open reading frame (ORF) of MHZ5 was amplified using PCR and cloned into the binary vector pCAMBIA2300-3SS-OSCs at the sites of KpnI and SalI.

To inhibit expression of the SL-relevant genes D17 and D3, D17-RNA interference (D17-RNAi) and D3-RNA interference (D3-RNAi) vectors were constructed as follows. A 375-bp fragment of the D17 ORF and a 438-bp fragment of the D3 ORF were cloned in both orientations in pCAMBIA2300Actin at the sites Sall and BamHI and separated by the first intron of the G20 oxidase of potato (Solanum tuberosum) to form a hairpin structure (Lo et al., 2005). All of the primers that were used above in this study are listed in Supplemental Table 2.

The above constructs were transformed into mhz5-3 or the wild type (Nipponbare) as previously described (Wu et al., 2009). The transformants were selected via PCR using kanamycin resistance (HPT II) gene-specific primers (Supplemental Table 2). Homozygous T3 or T4 transgenic lines were selected via kanamycin treatment (50 mg/L).
Measurement of ABA, Ethylene, and SL Production

For the ABA content assays, 3-d-old wild-type and mh25-1 etiolated seedlings were treated with or without 10 ppm ethylene for 24 h, and the shoots (containing the coleoptile and the first leaves) and roots were harvested. For each sample, ~200 mg of fresh tissue was homogenized under liquid nitrogen, weighed, and extracted for 24 h with cold methanol containing antioxidant and 6 ng H4-ABA (internal standard; OlChemim). Endogenous ABA was purified and measured as previously described (Fu et al., 2012) with some changes in detection conditions. The ultra-performance liquid chromatography-tandem mass spectrometer system consists of a UPLC system (ACQUITY UPLC; Waters) and a hybrid triple quadrupole-linear ion trap mass spectrometer (QTRAP 5500; AB SCIEX). The chromatographic separation was achieved on a BEH C18 column (50 mm × 2.1 mm, 1.7 μm; Waters) with the column temperature set at 25°C and a flow rate of 0.2 mL/min. The linear gradient runs from 95 to 85% A (solvent A, 0.05% acetic acid aqueous; solvent B, acetonitrile) in 1 min, 85 to 20% A in the following 1 min, and reequilibrated with the initial condition for 2 min. The optimized mass spectrometer parameters were set as follows: curtain gas 40 p.s.i., collision gas 6 p.s.i., ion spray voltage −4300 V, and temperature 550°C. The declustering potential was −85 V and collision energy was −15 V. Multiple reaction monitoring (MRM) mode was used for quantification, and the selected MRM transitions were 262 > 153.1 for ABA and 268.1 > 159.3 for H4-ABA.

For the ethylene production assays, the seedlings were grown in the dark or under continuous light in a 40-ml Uncapped vial for 7 d at 28°C, after which the vials were sealed with a rubber syringe cap for 17 h, and 1 mL of headspace of each vial was measured using gas chromatography (GC-2014; Shimadzu). The ethylene production of the seedlings that were treated with AVG (50 μM) was measured in the same manner.

The SL collection, purification, and analysis were performed as previously described (Jiang et al., 2013) with some changes in detection conditions. SL was analyzed using the ultra-performance liquid chromatography-tandem mass spectrometer system consisting of a UPLC system (ACQUITY UPLC) equipped with a BEH C18 column (100 mm × 2.1 mm, 1.7 μm; Waters) and a hybrid triple quadrupole-linear ion trap mass spectrometer (QTRAP 5500; AB SCIEX) equipped with an electrospray ionization source. The gradient started from 50% mobile phase A (0.05% acetic acid in water) and increased mobile phase B (0.05% acetic acid in acetonitrile) from 50 to 90% in 5 min at 25°C with a flow rate of 0.3 mL/min. MS parameters were set as follows: ion spray voltage, 4500 V; desolvation temperature, 600°C; nebulizing gas, 50 p.s.i.; desolvation gas, 55 p.s.i.; curtain gas, 40 p.s.i.; declustering potential, 90 V; entrance potential, 85 V; collision cell exit potential, 12 V; and collision energy, 23 V. MRM mode was used for quantification, and the selected MRM transitions were 331.2 > 216.1 for epi-SDS and 337.2 > 221.1 for d5-epi-SDS.

For ABA, ethylene, and SL detection, each experiment was repeated three times, and the averages and standard deviations are shown.

Coleoptile Elongation and Root Growth Tests

For the ABA rescue of mh25-1 short roots, 1.5 liters of 0.04 μM ABA (mixed isomers; Sigma-Aldrich) was used. For the ABA rescue of mh25-1 ethylene sensitivity, seedlings were placed on a stainless steel sieve in a 5.5-liter air-tight plastic box. Then, 1.5 liters of solution with or without 0.1 μM ABA was added to the plastic box. ABA stock solutions were prepared in ethanol, and equivalent volumes of ethanol were added to the control. The seedlings were treated with or without ethylene (10 ppm) at 28°C in the dark. The coleoptiles of the wild type and mh25-1 were sprayed once daily with 0.1 μM ABA (containing 0.001% Tween 20) after germination. For the AVG treatment, the germinated seeds of the wild type and mh25-1 were placed on eight layers of cheesecloth that were immersed in solution in Petri dishes and incubated in a 5.5-liter air-tight plastic box. AVG was dissolved in water, and a final concentration of 50 μM was used to inhibit endogenous ethylene. The seedlings were treated with a range of concentrations of ethylene at 28°C in the dark. After 2.5 d of treatment, the coleoptile elongation and/or root growth were measured.

GR24 and Flu Treatment

Germinated rice seeds were placed on cheesecloth on a stainless steel sieve that was placed in a 5.5-liter air-tight plastic box and incubated at 28°C in the dark. The seeds were subjected to the following treatment. The air-tight plastic box contained 700 mL of water with either 0.25 μM Flu (Sigma-Aldrich) or 1 μM GR24, a synthetic SL analog (Chiralix). A preliminary experiment showed that Flu can substantially inhibit ABA biosynthesis in the shoots/coleoptiles and roots of etiolated rice seedlings (Supplemental Figure 11). Flu and GR24 were dissolved in acetone. The control treatments also contained 0.5% acetone. The ethylene treatment was performed as previously described (Ma et al., 2013).

Gene Expression Analysis Using RT-PCR

Three-day-old etiolated seedlings were treated for up to 8 h with 10 ppm ethylene or air or with the application of 100 μM ABA and/or 100 μM NDGA with or without ethylene. Equivalent volumes of ethanol were added to the ABA-free or NDGA-free controls. After treatment, the shoots and roots were harvested and immediately frozen in liquid nitrogen. The total RNA extraction and RT-PCR were performed as previously described (Ma et al., 2013). Rice Actin1 and Actin2 was used as the internal control to quantify the relative level of each target gene. The gene-specific primers are listed in Supplemental Table 2.

Genetic Analysis

Double mutants of ers1 mh25-1, ers2 mh25-1, etr1 mh25-1, mh25-3 EIN2-3, and ein2 MHZ5-48 were generated by crossing their respective parental lines and identified by genotyping from their F2 populations, respectively, and their progeny were phenotypically and/or genotypically analyzed in F3 or F4 populations.

Agronomic Trait Analysis

The germinated seeds were grown on a stainless steel sieve in Kimura nutrient solution in a climate chamber. Two weeks later, the maximum length and the number of primary roots, adventitious roots, and lateral roots were measured. After harvest, agronomic traits, including the well-filled grain length/width, the number of primary and second branches per panicle, and the tiller number of mh25 and the wild type were analyzed.

Statistical Analysis

The relative root or coleoptile length is analyzed relative to the length of each genotype in untreated conditions. To analyze the gene expression level, each gene expression level in untreated wild type was set to 1. All of the data were analyzed using a one-way ANOVA (LSD t test) for the test groups with SPSS 18.0.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: Os01g36440 (MHZ5), Os07g06130 (EIN2), Os10g36650 (Actin2), Os08g13440 (Germin-like), Os09g11480 (EF063), Os09g11460 (EF073), Os07g03600 (Photosystem II 10 kD polypeptide), Os08g09080 (RGL1), Os06g08340 (ERF022), Os06g07040 (AA20), Os11g05740 (RPP28), Os07g26720 (RPA4), Os09g27750 (AC03), Os05g05680 (AC05), Os04g48850 (AC52), Os06g03990 (AC56), Os04g46470 (D17), Os06g06050 (D3), Os03g08585 (Actin1), Os03g06900 (EFP1), Os05g06320 (EFP2), and Os04g08740 (EFP2). mh25-4 represents a Tcoc17 insertion mutant (NG0489). ers1 represents a
T-DNA insertion mutant (1B-08531), en2 represents a T-DNA insertion mutant (3A-14390). ett2 represents a T-DNA insertion mutant (4A-03543).

Supplemental Data

Supplemental Figure 1. Phenotype of the mhz5 Mutant in the Field.

Supplemental Figure 2. Comparison of Root Development between Wild-Type and mhz5-1 Mutant Plants.

Supplemental Figure 3. Phenotype and Ethylene Response of mhz5-4.

Supplemental Figure 4. Pigment Analysis of Wild-Type and mhz5-1 Roots.

Supplemental Figure 5. Greening Phenotype and Chlorophyll Accumulation of the Wild Type and mhz5-1 Mutant.

Supplemental Figure 6. GR24 Cannot Rescue the Ethylene Response of mhz5-1.

Supplemental Figure 7. ABA Dose-Response Curves for Wild-Type Coleoptile and Root.

Supplemental Figure 8. Effect of AVG on Ethylene Production and the Coleoptile Ethylene Response of the Wild Type and mhz5-1 Mutant.

Supplemental Figure 9. Characterization of the Ethylene Receptor Mutants of Rice.

Supplemental Figure 10. Ethylene Response of Other ABA-Deficient Mutants in Rice.

Supplemental Figure 11. Quantification of the ABA Levels in Flu-Treated Wild-Type Seedlings.

Supplemental Table 1. Primers Used for Receptor Mutant Analysis via PCR-Based Genotyping.

Supplemental Table 2. Primers Used for Gene Expression Analysis and Vector Construction.

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Ethylene, Carotenoids, and ABA in Rice


Ethylene Responses in Rice Roots and Coleoptiles Are Differentially Regulated by a Carotenoid Isomerase-Mediated Abscisic Acid Pathway

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