Ethylene-Induced Inhibition of Root Growth Requires Abscisic Acid Function in Rice (Oryza sativa L.) Seedlings

Biao Ma1*, Cui-Cui Yin1*, Si-Jie He1, Xiang Lu1, Wan-Ke Zhang1, Tie-Gang Lu2, Shou-Yi Chen1*, Jinsong Zhang1*

1 State Key Lab of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, 2 Biotechnology Research Institute/National Key Facility for Genetic Resources and Gene Improvement, Chinese Academy of Agricultural Sciences, Beijing, China

Abstract

Ethylene and abscisic acid (ABA) have a complicated interplay in many developmental processes. Their interaction in rice is largely unclear. Here, we characterized a rice ethylene-response mutant mhz4, which exhibited reduced ethylene-response in roots but enhanced ethylene-response in coleoptiles of etiolated seedlings. MHZ4 was identified through map-based cloning and encoded a chloroplast-localized membrane protein homologous to Arabidopsis thaliana (Arabidopsis) ABA4, which is responsible for a branch of ABA biosynthesis. MHZ4 mutation reduced ABA level, but promoted ethylene production. Ethylene induced MHZ4 expression and promoted ABA accumulation in roots. MHZ4 overexpression resulted in enhanced and reduced ethylene response in roots and coleoptiles, respectively. In root, MHZ4-dependent ABA pathway acts at or downstream of ethylene receptors and positively regulates root ethylene response. This ethylene-ABA interaction mode is different from that reported in Arabidopsis, where ethylene-mediated root inhibition is independent of ABA function. In coleoptile, MHZ4-dependent ABA pathway acts at or upstream of OsEIN2 to negatively regulate coleoptile ethylene response, possibly by affecting OsEIN2 expression. At mature stage, mhz4 mutation affects branching and adventitious root formation on stem nodes of higher positions, as well as yield-related traits. Together, our findings reveal a novel mode of interplay between ethylene and ABA in control of rice growth and development.

Introduction

The gaseous phytohormone ethylene regulates many aspects of plant growth and development, including seed germination, seedling growth, flower translocation, sex determination, fruit ripening, organ senescence/abscission and adaptive responses to multiple biotic and abiotic stresses [1]. Ethylene is synthesized from methionine via a simple linear pathway, in which 1-aminocyclopentane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) function as key enzymes [2]. Ethylene is perceived by a family of membrane-bound receptors that have similarity to bacterial two-component histidine kinase and act as negative regulator of ethylene response [3]. Ethylene signal is transmitted via a linear signaling cascade that consists of CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), ETHYLENE INSENSITIVE 2 (EIN2) and EIN3/EIN3-LIKE (EIL) [4–6]. Ethylene receptors, CTR1 and EIN2 are all predominantly localized at the endoplasmic reticulum membranes on where they can form signaling complex [7–10]. Without ethylene, the receptors are believed to be active and activate CTR1 ser/thr protein kinase which in turn phosphorylates the positive regulator EIN2, likely causing the proteasomal degradation of EIN2 by F-box proteins EIN2 TARGETING PROTEIN1/2 (ETP1/2) [11–13]. Upon ethylene perception, EIN2 can be proteolytically cleaved and its carboxyl terminus is translocated into the nucleus, triggering EIN3/EILs-mediated transcriptional cascades to induce various ethylene responses [12,14,15]. EIN3/EILs are also subjected to proteasomal degradation mediated by EIN3-BINDING F-BOX PROTEIN1/2 (EBF1/2) [16,17].

In most growth and developmental processes, ethylene achieves its function through interaction with other phytohormones [18–20]. Among them, ethylene extensively interacts with abscisic acid (ABA) in many biological processes [21–29]. ABA plays pivotal roles in seed dormancy and germination, seedling development, stomatal closure and adaptive stress responses. ABA is produced from carotenoids, and the direct precursors are xanthophylls [30]. In Arabidopsis, zeaxanthin is converted into violaxanthin by Zeaxanthin epoxidase AB1 [31]. ABA4 is involved in the subsequent conversion of zeaxanthin to neoxanthin although no enzyme activity was identified [32]. In addition to ABA4, recent study revealed that NXd1 (Neoxanthin-Deficient 1) is also necessary for neoxanthin synthesis but does not affect ABA
Author Summary

Rice is a monocotyledonous plant that is distinct from the dicotyledonous model plant Arabidopsis in many aspects. In Arabidopsis, ethylene-induced root inhibition is independent of ABA action. In rice, however, we report here that ethylene inhibition of root growth requires ABA function. We identified MHZ4, a rice homolog of Arabidopsis ABA4 that is involved in ABA biosynthesis. The mhz4 mutant displayed reduced ABA level and exhibited ethylene-hypoosensitive root, but hypersensitive coleoptile phenotypes in etiolated seedlings. Exogenous application of ABA largely recovered the defective ethylene responses. Overexpression of MHZ4 resulted in enhanced and reduced ethylene response in the roots and coleoptiles, respectively. In root, MHZ4-dependent ABA pathway genetically acts at or downstream of ethylene receptors and positively regulates root ethylene response. Moreover, ethylene treatment stimulated ABA production in roots at least through transcriptional activation of MHZ4. The results indicate that ethylene-induced root inhibition in rice is largely mediated through MHZ4-dependent ABA function. In coleoptile, MHZ4-dependent ABA pathway acts at or upstream of OsEIN2 and negatively regulates coleoptile ethylene response, possibly via transcriptional suppression of OsEIN2. Together, our findings reveal a novel mode of ethylene-ABA interaction which is fundamentally different from that in Arabidopsis.

Ethylene Requires Abscisic Acid for Root Inhibition in Rice

Ethylene-induced root inhibition is dispensable for ABA action [21,22]. Although the integration of ethylene and ABA signaling pathways has been elucidated in Arabidopsis, their interactions in other plants remain largely unclear.

Rice is an important crop worldwide. A few ethylene signaling components homologous to those of Arabidopsis have been characterized in rice including ethylene receptor OsETR2, OsRTH1, OsCTR and OsEIN2 [40–44]. Recently, through analysis of rice ethylene-response mutant mhz7, we find that MHZ7/OsEIN2 plays central roles in ethylene signalling and regulation of agronomic traits in rice, and clear ethylene-insensitive and hypersensitive phenotypes are identified in etiolated rice seedlings [45]. In this study, we further characterized another rice ethylene-response mutant mhz4 (miao huya2, Chinese name with a English meaning of cat whisker), which displays reduced ethylene-response in roots but enhanced ethylene-response in coleoptile [45]. Through map-based cloning, the MHZ4 was identified to encode a membrane protein orthologous to Arabidopsis ABA4, which is responsible for the conversion of zeaxanthin to neoxanthin in ABA biosynthesis pathway [32]. Mutation of MHZ4 abolishes ABA production but promotes ethylene emission. MHZ4 overexpression enhances root ethylene-response but reduces coleoptile ethylene-response. MHZ4 acts downstream of ethylene receptors in regulating root growth but upstream of OsEIN2 in regulating coleoptile elongation. Our results reveals the complicated interplay between ethylene and ABA signaling in regulating rice seedling growth and agronomic traits, providing new insight into understanding of their interaction in rice.

Results

Characterization of mhz4 Mutant for Ethylene Response Phenotypes

The mhz4 is identified previously in our screen for ethylene-response mutants in rice [45]. For dark-grown wild type (WT) seedlings, ethylene inhibited root growth but promoted coleoptile elongation in a dose-dependent manner (Figure 1A-C). The roots of etiolated mhz4 seedlings were about 10% shorter than that of WT seedlings under normal conditions. Upon ethylene treatment, mhz4 roots were insensitive to ethylene inhibition at lower concentrations (≤1 ppm) but displayed mild growth inhibition at higher concentrations (10 to 100 ppm), e.g. about 20% inhibition in mhz4 compared with about 70% inhibition in the WT at 10 ppm ethylene (Figure 1A and 1B). This indicates that the roots of mhz4 are less sensitive to ethylene. On the other hand, the coleoptiles of mhz4 were slightly but significantly (P<0.004) longer than that of WT seedlings in the absence of ethylene and were much longer than that of WT under all concentrations of ethylene treatment, indicating that the coleoptiles of mhz4 are hypersensitive to ethylene (Figure 1A and 1C). These results indicate that mhz4 mutation oppositely affects ethylene responses in roots and coleoptiles of etiolated rice seedlings.

To further confirm the ethylene responsiveness of mhz4 mutant, we examined expressions of ethylene-inducible genes originally identified from a chip analysis (GSE51153; [45]) and a RNA-seq analysis (SRP041468). Two genes including disease resistance gene and peptidylprolyl isomerase gene were found to be induced by ethylene in both shoots and roots of WT seedlings (Figure 1D). Four genes (OsERF002, OsRR45, OsRAP2.8 and OsIAA20) were found to be induced mainly in roots and four genes (SHR5, OsERF063, OsERF073 and photosystem II 10 kDa polypeptide gene) mainly in shoots of WT seedlings (Figure 1E). In mhz4 shoots, the ethylene inducible genes were...
Figure 1. Ethylene responses in etiolated *mhz4* seedlings. Rice seedlings were grown in dark for 3 d in the presence of various concentrations of ethylene. (A) Ethylene-response phenotypes of WT (Nipponbare) and *mhz4* seedlings grown in air or 10 ppm ethylene. Bar = 10 mm. (B) Ethylene dose-response curves for root length (top) and relative root length (bottom) in WT and *mhz4* seedlings. Each point is average of 20 to 30 seedlings and bars indicate SD. (C) Ethylene dose-response curves for coleoptile length (top) and relative coleoptile length (bottom) of WT and *mhz4* seedlings. Others are as in (B). (D) Expression of ethylene-inducible genes in both shoot and root of WT and *mhz4* seedlings. Dark-grown 2 d-old seedlings were treated with or without 10 ppm ethylene for 8 h and the RNA was isolated for quantitative PCR. Data are the mean ± SD of four replicates. (E) Expression of ethylene-inducible genes in root (left) or shoot (right) of WT and *mhz4* seedlings. Others are as in (D).

doi:10.1371/journal.pgen.1004701.g001
constitutively expressed at a level higher than that in ethylene-treated WT. Ethylene treatment of mhz4 did not further increase
the transcript levels probably due to their expression levels were
already very high. It should be noted that we used shoots instead of
coleoptiles for the gene expression analyses because similar
ethylene responses were found for coleoptiles and shoots [46]. In
mhz4 roots, the transcripts of ethylene inducible genes remained at
a similar or lower level when compared to those in WT in the
absence of ethylene, and these genes showed no or only slight
inductions compared to their ethylene inductions in WT roots
(Figure 1D, E). These results indicate enhanced and reduced
ethylene response in mhz4 shoots/coleoptiles and roots, respecti-
vately, at gene expression level.

The MHZ4 Gene Is Homologous to Arabidopsis ABA4

Genetic analysis has revealed that mhz4 is a recessive mutation
controlled by a single locus [45]. The MHZ4 gene was identified
by a map-based cloning approach using F2 plants from a cross
between mhz4 and indica rice variety Minghui 63. A total of 480
segregated mutant individuals were used for positional mapping of
the mhz4 locus. The mutation site was narrowed down to a 30-kb
region in chromosome 1 between Id1-1.55 and Id1-1.58
markers (Figure 2A). We sequenced all four annotated genes
within this region and found an 18-bp deletion in LC-
C_Os01g03750. The deletion occurred at the third intron and
disrupted the splicing site, resulting in 81-bp un-spliced intron
and causing a premature stop codon in the encoded protein (Figure 2A
and 2B). The mutations were further confirmed by PCR through
examination of fragment length polymorphisms in genomic DNA
cDNA of WT and mhz4 (Figure 2C). To verify that the
mutation of LOC_Os01g03750 locus is responsible for the mutant
phenotype of mhz4, we cloned the 4213-bp DNA fragment
including the complete LOC_Os01g03750 genomic sequence
(1147-bp) plus 2048-bp region upstream of the start codon and
858-bp extension downstream of the stop codon from the WT,
and transformed the gene into the mhz4 plants. Ethylene response
assays showed that the altered ethylene responsiveness of mhz4 was
rescued in the transgenic plants (Figure 2D). These results confirm
that MHZ4 is located at LOC_Os01g03750 locus.
The MHZ4 gene encodes a protein of 228 amino acids that
harbors an N-terminal signal peptide and four transmembrane
domains as predicted by the SMART program (http://smart.
embl-heidelberg.de/) (Figure 2B). The MHZ4 sequence shared
47% identity and 64% similarity with Arabidopsis ABA4
(At1G67080), which is required for neoxanthin formation in the
photosynthetic cells [47]. Phylogenetic analysis revealed that Mhz4 protein is conserved
from cyanobacteria to higher plants and is more closely related to homologues from monocotyledonous plants (Figure 2E).

MHZ4 Expression and Protein Subcellular Localization

MHZ4 accumulation was examined by semiquantitative RT-
PCR. The transcripts were detected in all organs from vegetative
to reproductive stages and found to be more abundant in young
leaves (Figure 3A). Transgenic rice plants harboring MHZ4
promoter::β-glucuronidase (GUS) construct were also generated
and GUS staining assay was performed to evaluate the promoter
activity. In etiolated seedlings, MHZ4 expression was detected in
both roots and coleoptiles, and the signals were also present in the
vascular tissues of roots (Figure 3B, a to e). In root apexes, GUS
signals were observed in the putative quiescent center (QC) and
root caps (Figure 3B, f). In field-grown plants, MHZ4 was
expressed in leaf blades, young stem nodes, the base of axillary
buds and adventitious roots derived from the nodes (Figure 3B, g
to j). In reproductive organs, the expression of MHZ4 was
detected in the anthers and pistil of young flowers, lemma of
mature flowers, and parts of developing grains (Figure 3B, k to n).
The MHZ4 protein was predicted to localize to the chloroplasts
by using the ChloroP 1.1 program (http://www.cbs.dtu.dk/ services/ChloroP/). To experimentally verify the localization,
MHZ4 coding sequence was fused in frame with GFP and
transiently expressed in tobacco leaves. The fluorescence signals
of MHZ4-GFP fusion protein were found in the chloroplasts of
tobacco glandular hairs, as identified by co-localization with the
chlorophyll autofluorescence (Figure 3C). This result is consistent
with the localization of Arabidopsis ABA4 in chloroplast envelope
[47].

Ethylene-Induced Root Inhibition in Rice is Largely
Mediated through MHZ4-Dependent ABA Accumulation

Since Mhz4 is homologous to Arabidopsis ABA4 in ABA
biosynthesis, we tested whether endogenous ABA contents in mhz4
were altered. The mhz4 mutant contains 61% and 7.0% of WT
ABA levels in roots and shoots, respectively, demonstrating that
ABA production is severely hampered in mhz4 mutant (Fig. 4A).
Ethylene induced ABA accumulation in WT roots but not in
shoots, suggesting organ-specific regulation of ABA accumulation
(Figure 4A, top). Ethylene induction of ABA was not observed in mhz4
roots (Fig. 4A), indicating that MHZ4 is required for ethylene-
induced ABA accumulation. We further examined the expression of ABA-responsive gene OsMFT2 (LOC_Os01g02120) previously
identified by Lenka et al. [48]. OsMFT2 expression was dramatically induced by ABA in both roots and shoots of WT
seedlings (Figure 4B, top). However, the transcripts were barely
detectable in mhz4 mutant compared with those in WT
(Figure 4A, bottom). These results indicate that MHZ4 is
responsible for ABA biosynthesis in rice.

Under normal growth condition, the mhz4 roots of etiolated
seedlings are slightly but significantly shorter than the WT roots
(Figure 4C). Treatment of the seedlings with 0.04 μM ABA
completely restored the short root phenotype (Figure 4C),
suggesting that basal levels of endogenous ABA are required for
the maintenance of normal root elongation. The same ABA
concentration showed no obvious stimulation on root growth of
WT seedlings (Figure 4C).

Considering that MHZ4 mutation leads to the reduced ethylene
sensitivity in mhz4 roots, we investigated whether addition of ABA
could rescue the ethylene response of the mutant. The 0.1 μM
ABA was used in the complementation assay because at this
concentration, no obvious inhibitory effects were observed on root
growth in WT seedlings (Figure S1). In the presence of 10 μM
ACC (precursor of ethylene), application of 0.1 μM ABA largely
rescued the defective response of mhz4 roots to ethylene
(Figure 4D and 4E), indicating that reduced ethylene sensitivity
of mhz4 roots is most likely caused by the lack of ABA.

We further examined MHZ4 expression in response to
ethylene. MHZ4 transcripts were significantly induced by ethylene
in WT roots and shoots (Figure 4F). Promoter-GUS analysis also
showed that ethylene treatment stimulated MHZ4 promoter
activity mainly in the vascular tissues of roots (Figure 4G). These
results suggest a role for MHZ4 in root growth control. It should
be noted that although MHZ4 expression was also induced by
ethylene in shoots, the ABA level did not increase after ethylene
treatment (Figure 4A and 4F). This is likely owing to ethylene-
activated ABA catabolism for a homeostasis in shoots [23,30,49].

To further elucidate the role of ethylene-triggered ABA in root
ethylene response, we investigated the expression of ethylene-
inducible genes in response to ABA as well as ABA biosynthesis
inhibitor. OsIAA20 transcripts were dramatically induced not only by ethylene but also by ABA in rice roots (Figure 4H). However, ethylene induction of OsIAA20 expression was abolished in the presence of NDGA (nordihydroguaiaretic acid), an ABA biosynthesis inhibitor that specifically inhibits NCED enzyme activity (Figure 4H). These results suggest that ethylene-induced ABA mediates expression of some ethylene-responsive genes. Taken together, these findings suggest that ethylene-induced root growth inhibition is largely mediated through MHZ4-dependent ABA accumulation.

Figure 2. Map-based cloning of mhz4 locus. (A) Fine mapping of MHZ4 gene. The locus was mapped to chromosome 1 within a 30 Kb region between IdI1–1.55 and IdI1–1.58 markers. Mutation site is indicated in schematic diagram of MHZ4 gene. Black boxes represent exons. (B) Schematic structure of the MHZ4 protein. The structure is predicted using the SMART software (http://smart.embl-heidelberg.de). Mutation site is shown. cTP indicates chloroplast transit peptide identified using the ChloroP 1.1 program (http://www.cbs.dtu.dk/services/ChloroP/). Blue columns represent transmembrane domains. (C) Confirmation of mhz4 mutation site in both genomic DNA and cDNA by PCR. M: marker. (D) Functional complementation of mhz4 mutant. MHZ4 genomic DNA (4213 bp) was transformed into mhz4 plants (MHZ4/mhz4), rescuing the enhanced coleoptile elongation and reduced root inhibition phenotypes of mhz4 in the presence of ethylene (10 ppm). Bottom is confirmation of the transgene by PCR using the genomic DNA as templates. Bar = 10 mm. (E) Phylogenetic analysis of MHZ4/OsABA4 and its homologous proteins from other plants. The phylogenetic tree is generated by Maximum Likelihood method. Accession numbers are as follows: Vitis vinifera, XP_002283875; Populus trichocarpa, XP_002305164; Ricinus communis, XP_002521222; Citrus sinensis, ADH82117; Glycine max, XP_003532342; Medicago truncatula, XP_003618901; Arabidopsis thaliana, NP_564889; Brachypodium distachyon, XP_003565288; Setaria italica, XP_004968073; Zea mays, ACN29324; Physcomitrella patens, Pp1s108.75V6; Anabaena cylindrica, WP_015215835; Synechocystis, BAA18538.
Ethylene-Hypersensitivity of *mhz4* Coleoptiles Is Caused by Enhanced Ethylene Signaling

The *mhz4* coleoptiles showed enhanced ethylene response (Figure 1A, C). We determined whether ABA addition would complement the mutant response to ethylene. Without ABA, the coleoptile length of *mhz4* mutant was significantly longer than that of WT seedlings in the presence or absence of ethylene (Figure 5A and 5B). When 0.1 μM ABA was applied to roots, however, the *mhz4* coleoptiles were identical in length to that of WT seedlings with or without ethylene treatment (Figure 5A and 5B). These observations suggest that application of 0.1 μM ABA substantially restored the *mhz4* coleoptile ethylene response to WT levels and that ABA-deficiency is responsible for the ethylene hypersensitivity of *mhz4* coleoptiles.

Enhanced ethylene response can be caused by ethylene overproduction and/or enhanced signal transduction. We then measured ethylene production in *mhz4*. The *mhz4* mutant produced 2 to 12 times more ethylene than WT and application of 1 μM ABA to etiolated *mhz4* seedlings dramatically reduced the ethylene levels, suggesting that ABA-deficiency in *mhz4* mutant promote ethylene biosynthesis (Figure 5C). Furthermore, quantitative PCR analysis revealed that the ethylene biosynthetic genes...
Figure 4. Ethylene-induced root inhibition is largely mediated through MHZ4-dependent ABA accumulation. (A) ABA levels in WT and mhz4 seedlings in the absence or presence of ethylene. Two-day-old etiolated seedlings were treated with or without 100 ppm of ethylene for 48 h. Data are the mean ± SD of three replicates. * and ** indicate significant difference between the compared two samples at P<0.05 and P<0.01, respectively. (B) Expression of ABA-responsive gene OsMFT2 (LOC_Os01g02120). Upper, OsMFT2 transcripts in 3 d-old etiolated seedlings of WT in response to ABA (100 μM, 6 h). Bottom, OsMFT2 transcripts detected in mhz4 and WT etiolated seedlings. Actin1 was used as an internal control. (C) Rescue of the root length of mhz4 by ABA. Rice seedlings were grown in the dark for 2.5 days in the presence or absence (Mock) of 0.04 μM ABA. Each column is average of 40 seedlings and bars indicate SD. Different letters above each column indicate significant difference between the compared pairs (P<0.01). (D) Rescue of the reduced ethylene sensitivity of mhz4 roots by ABA. WT and mhz4 seedlings were grown in the dark for 2.5 d in the absence or presence of 10 μM ACC, with or without supplementation of 0.1 μM ABA. Bar = 10 mm. (E) Quantification of root inhibition in (D). Each column is average of 40 seedlings and bars indicate SD. ** indicates significant difference between the linked two samples at P<0.01. (F) Quantitative PCR analysis of MHZ4 expression in response to ethylene. WT seedlings were grown in the dark for 2.5 d and then treated with 10 ppm ethylene for 0–6 h. Data are the mean ± SD of four replicates. ** indicate significant difference compared to 0 h at P<0.01. (G) Ethylene-induced GUS activity in roots of transgenic plants harboring MHZ4pro::GUS construct. One-day-old etiolated seedlings were treated with or without 10 ppm ethylene for 24 h. The roots were cut off and stained for GUS activity for two days. Twenty to thirty roots were observed for each treatment and representative samples are presented. Bar = 1 mm. (H) Quantitative PCR analysis of OsIAA20 expression in response to ABA or ethylene+NDGA. Dark-grown 2 d-old WT seedlings were treated with 100 μM ABA for 6 h, or treated with 10 ppm ethylene for 8 h in the presence or absence (Mock) of 100 μM NDGA. The RNA from roots was isolated for quantitative PCR. Data are the mean ± SD of four replicates.

doi:10.1371/journal.pgen.1004701.g004
OsACS2, OsACS6, OsACO3 and OsACO5 were all elevated in mhz4 shoots, likely contributing to the enhanced ethylene production (Figure 5D).

We next investigated the contribution of ethylene overproduction to the enhanced ethylene response of mhz4 coleoptiles through treatment with the ethylene biosynthesis inhibitor 1-
aminoethoxyvinyl-glycine (AVG). Application of 5 µM AVG removed most of the increased ethylene production in mhz4 mutant, but exerted limited effect on the coleoptile ethylene response (Figure 5E and 5F, Figure S2). This result suggests that ethylene overproduction is not the main reason for the enhanced ethylene response in mhz4 coleoptiles. We further examined the possibility of ethylene signaling and found that OsEIN2 transcripts were particularly enriched in mhz4 shoots compared with that of WT (Figure 5G). These results suggest that the enhanced ethylene response of mhz4 coleoptiles is most likely caused by enhanced ethylene signaling.

MHZ4 Overexpression Alters Ethylene Response in Roots and Coleoptiles

To further study the function of MHZ4 in rice ethylene response, we transformed the gene into WT rice plants under the control of the CaMV 35S promoter. MHZ4-overexpressing (MHZ4-OX) lines were identified by semiquantitative RT-PCR (Figure S3), and four representative lines were used for further analysis. The four transgenic lines all showed slightly but significantly shorter roots (P<10−5) and coleoptiles (P<10−5) in the dark compared with WT seedlings, indicating constitutive growth inhibition of the roots and coleoptiles in MHZ4-OX plants (Figure 6A–6C). Upon ethylene treatment, the MHZ4-OX lines exhibited strong inhibition of root growth but less promotion of coleoptile growth in comparison with WT seedlings, indicating enhanced ethylene response in the roots but reduced response in the coleoptiles of MHZ4-OX lines (Figure 6A–6C). Examination of the ethylene-inducible gene OsERF002 in the roots and SHR3 in the shoots further proved the ethylene responsiveness of MHZ4-OX lines (Figure 6D). Additionally, in the shoots, OsEIN2 transcript levels were down-regulated in MHZ4-OX lines compared with that in WT seedlings (Figure 6E), suggesting that the reduced ethylene responsiveness of the coleoptiles is likely related to the reduction of ethylene signaling. Together, these results indicate that MHZ4 overexpression leads to different ethylene responses in roots and coleoptiles.

Genetic Interactions of MHZ4 and Ethylene Signaling Pathway

We further examined the genetic relationship of MHZ4 and ethylene signaling pathway through double mutant analyses. A loss-of-function Oser1 mutant was obtained from the POSTECH Biotech Center [50] and was identified by PCR-based analyses (Figure S4). The roots of Oser1 etiolated seedlings were significantly shorter than that of WT seedlings in the absence of ethylene and displayed a strong ethylene response phenotype following treatment with 1 ppm ethylene, indicating the presence of enhanced ethylene response in Oser1 roots (Figure 7A and 7B). The roots of mhz4 Oser1 double mutant were slightly shorter than that of the mhz4 single mutant but exhibited ethylene insensitive response that was indistinguishable from that of mhz4, suggesting that OsERS1-mediated root ethylene response requires MHZ4 function (Figure 7A and 7B). The coleoptiles of Oser1 did not exhibit apparent ethylene response phenotype compared to that of WT (Figure 7A).

We constructed mhz4 Osein2 double mutant to analyze the genetic interaction of MHZ4 with OsEIN2 in coleoptile ethylene response. The Osein2/mhz7-1 is a mutant showing ethylene insensitivity in both roots and coleoptiles previously identified [45]. The coleoptiles of mhz4 Osein2 double mutant showed complete ethylene insensitivity similar to that of Osein2 seedlings but unlike the mhz4 coleoptiles, indicating that mhz4 requires OsEIN2 in regulation of coleoptile ethylene response (Figure 7C and 7D). On the other hand, the roots of mhz4 Osein2 double mutant showed complete ethylene insensitivity similar to the case in Osein2 roots (Figure 7E). Considering that mhz4 roots still had some ethylene response and this weak response was completely abolished in the double mutant, we propose that the residual ethylene response observed in mhz4 roots was dependent on OsEIN2 function.

Effects of MHZ4 on Plant Growth and Yield-Related Traits

We compared phenotypes of field-grown mhz4 mutant, MHZ4-OX lines and WT plants. At seedling stage, the mhz4 mutant had flowing leaves with yellow green color compared with WT plants (Figure 8A). From vegetative to reproductive stages, the leaves of mhz4 appeared to be pale green and some leaves had brown dots on the leaf tip (Figure 8B). Chlorophyll (Chl) analysis revealed that mhz4 had a reduction in Chl b and total Chl contents and an increase in Chl a/b ratio compared with WT leaves (Figure 8C), indicating that mhz4 mutation interferes with Chl b biosynthesis. This may alter the light harvesting antenna size, thus affecting photosynthetic efficiency [51, 52]. For flowering time, both mhz4 mutant and the four MHZ4-OX lines exhibited delayed heading time in comparison with WT plants (Table S2). At maturation stage, mhz4 mutant exhibited dramatic growth of branches developed on upper nodes and adventitious roots formation on the nodes in comparison with WT plants (Figure 8D–8F). Similar phenotype was observed in the mhz4 Osein2 double mutant in different years (Figure 8G–8I; Figure S5). These results suggest that MHZ4 negatively regulate axillary bud growth and adventitious rooting possibly through ABA function but not ethylene signaling.

Plant height is an important agronomic trait associated with rice yield. Ethylene positively regulates rice plant height by promoting stem elongation [53]. The mhz4 plants were taller than WT and all the four MHZ4-OX lines were shorter than WT plants (Table S2). The mhz4 Osein2 double mutant was shorter than mhz4 plants but was similar to that of Osein2 plants (Table S2). These observations suggest that MHZ4 negatively regulate plant height in rice in an OsEIN2-dependent manner. The promoted plant height of mhz4 is possibly due to the increased ethylene production (Figure 5C). The numbers of effective tiller (tillers producing panicles with at least five filled grains) in WT and mhz4 were identical, whereas two of the four MHZ4-OX lines produced more effective tillers than the WT (Table S2). Panicle length of mhz4 mutant was shorter than that of WT (Table S2). Grain-related traits were also examined. The mhz4 grains had no dormancy and displayed preharvest sprouting phenotype (Figure S6A and S6B). This is a typical characteristic for ABA-deficient mutants of rice [54]. Seed-setting rate and 1000-grain weight were all significantly reduced in mhz4 mutant (Table S2). The grain shape of WT and mhz4 was further examined. The mhz4 mutant showed a decrease in grain thickness and an increase in grain length, implying that MHZ4 mutation affects grain shape (Figure S6C). We further performed a time course analysis of grain dry weight during grain filling and found that the grain weight of mhz4 increased more slowly than that of WT, suggesting that grain filling is hindered in mhz4 mutant (Figure S6D). These results indicate that alteration of MHZ4 expression affects branching, plant height and grain-related traits.

Discussion

We characterized a rice ethylene-response mutant mhz4, which showed reduced ethylene response in roots but enhanced ethylene response in coleoptiles. MHZ4 encoded a chloroplast-localized...
membrane protein homologous to Arabidopsis ABA4 in ABA biosynthesis pathway. MHZ4 mutation reduced ABA level but enhanced ethylene production. MHZ4 overexpression enhanced ethylene responses in roots but reduced ethylene response in coleoptiles. Genetically, MHZ4 acts at or downstream of the ethylene receptors to positively regulate root ethylene response likely through ethylene-triggered ABA accumulation. Additionally, MHZ4 acts at or upstream of OsEIN2 to negatively regulate coleoptile ethylene response possibly through modulating OsEIN2 expression. MHZ4 also affects agronomic traits. Our findings reveal a novel mode of interplay between ethylene and ABA.

Figure 6. MHZ4 overexpression confers enhanced and reduced ethylene responses in roots and coleoptiles, respectively. (A) Ethylene response phenotypes in WT and MHZ4-overexpressing (MHZ4-OX) lines. Rice seedlings were grown in dark for 2.5 d in the presence or absence of 1 ppm ethylene. Bar = 10 mm. (B) Ethylene dose-response curves for root length (top) and relative root length (bottom) in WT and MHZ4-OX lines. (C) Ethylene dose-response curves for coleoptile length (top) and relative coleoptile length (bottom) in WT and MHZ4-OX lines. Others are as in (B). (D) Expressions of ethylene-inducible genes in roots (top) and shoots (bottom) of WT and MHZ4-OX lines. Dark-grown 2 d-old seedlings were treated with or without 10 ppm of ethylene for 8 h and the RNA was isolated for quantitative RT-PCR. Data are the mean ± SD of four replicates. (E) OsEIN2 gene expression in shoots of WT and MHZ4-OX lines detected by semi-quantitative PCR. Rice seedlings were grown in dark for 3 days and RNA was isolated from shoots. Actin1 was used as an internal control.

doi:10.1371/journal.pgen.1004701.g006
Figure 7. Genetic interactions of MHZ4 with ethylene receptor gene and MHZ7/OsEIN2 gene. (A) Comparison of ethylene-response phenotypes of mhz4 Osers1 double mutant and the single mutants. Nipponbare (Nip) and Dongjin (DJ) are wild types. Double mutant between mhz4 and ethylene receptor OsERS1 mutant Osers1 (Dongjin background) were generated by crossing. Rice seedlings were grown in the dark for 3 d in the presence or absence of 1 ppm ethylene. (B) Quantification of root length (left) and relative root length (right) of the mutants in (A). Each column is average of 50 seedlings and bars indicate SD. ** indicate significant difference compared to air control at P<0.01. (C) Ethylene response phenotypes of double mutant mhz4 Osein2 and the single mutants. Osein2/mhz7-1 is in Nipponbare background. Rice seedlings were grown in dark for 3 d in the presence or absence of 10 ppm ethylene. (D) Ethylene dose-response curves for coleoptile length in various mutants. Each point is average of 20 seedlings and bars indicate SD. (E) Ethylene dose-response curves for root length (left) and relative root length in various mutants (right). Others are as in (D). Bars = 10 mm.

doi:10.1371/journal.pgen.1004701.g007
The roots of *mhz4* seedlings displayed about 20% growth inhibition (80% insensitivity) when treated with 100 ppm ethylene (Figure 1B). By contrast, the WT roots exhibited about 70% inhibition under the same concentration of ethylene. This comparison reveals that *MHZ4* mediates a large part of the ethylene-inhibition of root growth. Since *MHZ4* mutation reduced ABA contents possibly through disruption of a branch of ABA biosynthesis (Figure 4A), we propose that ABA may be the factor participating in ethylene-inhibition of root growth. Five pieces of evidence support this conclusion. (1) Exogenous application of ABA largely recovered the defective response of *mhz4* roots to ethylene; (2) *MHZ4*-overexpression resulted in enhanced ethylene response in roots; (3) Ethylene-induced *MHZ4* expression and ABA accumulation particularly in roots; (4) Ethylene-induced ABA mediates expression of some ethylene-responsive genes; (5) The ethylene receptor *OsERS1*-mediated root ethylene response required *MHZ4* function. The present conclusion that ethylene inhibited root growth largely/partly through ABA function is in contrast to that observed in *Arabidopsis*, where ethylene-mediated root inhibition is independent of ABA action [21,22,26]. This difference indicates that rice, as a monocot or a semiaquatic plant, may have adopted a novel mechanism for ethylene-ABA interaction.

We have identified another rice ABA-deficient mutant *mhz5* that showed a similar ethylene response phenotype as *mhz4* and the corresponding gene *OsCRTISO* encodes the carotenoid isomerase that acts at early step in ABA biosynthetic pathway (Yin et al., unpublished). Consequently, it seems that the ABA-
dependent ethylene effect on rice root growth is not restricted to *MHZ4*, possibly other ABA biosynthetic genes and even the signaling component genes may affect the root ethylene response in rice.

In *Arabidopsis*, extensive studies have established that ethylene inhibits root growth through auxin action via modulating its biosynthesis, transport and/or signaling [53–62]. Auxin response occurring in the elongation zone mediates a substantial part of ethylene effect on root growth [63]. In monocotyledonous plant *Brachypodium*, ethylene may inhibit auxin biosynthesis in IPA pathway to control root elongation, which is different from the case in *Arabidopsis* [64]. In rice, our present results demonstrate that ethylene inhibits root growth largely through ABA function. Previous studies also discover that ethylene triggers ABA biosynthesis, leading to growth inhibition [65]. It is unknown whether auxin is also involved in this process. ABA may act through auxin pathway or vice versa, or the two hormones might act independently to mediate ethylene response. Further investigation toward the relationship among these hormones should shed light on their complicated interaction in control of rice roots.

*MHZ4* mutation drastically enhances ethylene emission possibly through activation of ethylene biosynthesis genes, and ABA inhibits ethylene production (Figure 5C). Considering that ethylene induces ABA to inhibit root growth (Figure 4), the ABA suppression of ethylene generation may represent a negative feedback control mechanism to alleviate ethylene effects on roots.

The coleoptiles of *mhz4* were slightly but significantly longer than that of WT seedlings (Figure 5B), indicating that ABA acts as an inhibitor of rice coleoptile elongation. Similar effect of ABA was observed in rice seedlings treated with fluridone, an inhibitor of ABA biosynthesis, which reduced the levels of endogenous ABA in coleoptiles and in turn promoted coleoptile growth [66]. In contrast to the ABA effect, ethylene stimulates coleoptile growth in rice (Figure 1; [46]), suggesting that the two hormones have antagonistic interaction. That *MHZ4* mutation enhanced ethylene production and ethylene response whereas its overexpression reduced ethylene response in coleoptiles (Figure 1, 5, and 6) further supports the antagonistic relationship of ABA and ethylene. In *Arabidopsis*, ABA biosynthesis gene *ABA2* mutation also resulted in elevated ethylene production [67]; however, ethylene response of this mutant was not altered [26,67], indicating presence of conserved and diverged aspects in interactions of ABA and ethylene.

In WT shoots, ethylene slightly inhibits ABA levels and may hence promote shoot coleoptile elongation (Figure 4A). In *mhz4* shoots, the ABA level is slightly induced by ethylene but the induction is not statistically significant (P = 0.089168). Additionally, the ABA level in *mhz4* shoots is quite low (about 1/10 of the WT level) compared with that in WT. The small increase in ABA contents in *mhz4* shoots after ethylene treatment may be not enough to affect its ethylene response. However, we could not exclude the possibility that ethylene-altered ABA levels may have some subtle effects on ethylene responses.

Although elevated ethylene production was observed in *mhz4* mutant, AVG treatments that inhibited ethylene production did not significantly altered ethylene response (Figure 5E), suggesting that the enhanced ethylene response in *mhz4* coleoptiles is likely caused by enhanced ethylene signaling rather than ethylene overproduction. In support of this prediction, transcript levels of the central ethylene signaling component *OsEIN2* were up-regulated in *mhz4* shoots (Figure 5G). Further genetic analysis revealed that *mhz4* ethylene-response phenotypes in coleoptiles required *OsEIN2* function. Collectively, *MHZ4*-dependent ABA pathway negatively regulates rice coleoptile ethylene response at least in part through modulating *OsEIN2* expression. It should be mentioned that although the ethylene overproduction in *mhz4* mutant did not play major roles in enhanced ethylene response, the basal level of ethylene did contribute to the coleoptile elongation because AVG reduced the coleoptile length of *mhz4* to the WT level (Figure 5E).

Our present results reveal that ABA and ethylene are synergistic in root growth inhibition but antagonistic in coleoptile elongation. The opposite effects of *mhz4* mutation on ethylene response of rice root and coleoptile may be due to alterations of expressions of ethylene-responsive genes in these organs (Figure 1D, E). Previous studies have reported antagonistic interactions of ethylene and ABA in several processes such as seed germination, stomatal closure and submerge-induced shoot elongation of semiaquatic plants, and synergistic interactions in tomato fruit ripening and abiotic stress responses [68]. These findings imply that ethylene-ABA interplay is quite complicated, depending on biological processes, tissue/organs, and plant species. Why plants have these different interactions remains unclear. It is possible that multiple interaction manners allow plants to be more adapted to the changing environments at different growth and developmental stages.

At mature stage, *mhz4* plants had branches and adventitious roots at nodes of higher positions (Figure 8). This phenomenon is rarely observed in rice and not dependent on *OsEIN2* function of ethylene signalling through double mutant analysis, suggesting that ABA itself inhibits branching and formation of adventitious root. This function probably represents a novel aspect of ABA roles during rice development. It has been reported that a higher ratio of ABA to ethylene in rice spikelets is required to maintain a faster grain-filling rate [69]. In our *mhz4* mutant, a low ABA level but a high ethylene production was noted and the low ratio of ABA to ethylene may be related to the impaired grain filling (Figure S6). However, in *MHZ4*-overexpressing plants, grain-related traits were not widely improved (Table S2), implying that these traits are likely regulated in a complicated manner.

The *MHZ4* seems to have a negative role on plant height because the *mhz4* mature plants are taller than WT whereas the overexpressing lines are shorter than WT (Table S2). *MHZ4* mutation activated ethylene production which increased plant height through *OsEIN2* function. Our results are similar with those obtained in *Arabidopsis* and tomato, where ABA-deficient mutants exhibit stunted shoot growth due to increased ethylene production [67,70]. It should be noted that ethylene promotes shoot growth in rice but inhibits this process in *Arabidopsis* and tomato.

In conclusion, we demonstrate that ethylene inhibits rice root growth through ABA action, and ABA negatively regulates coleoptile ethylene response through modulating *OsEIN2* expression. Synergistic interaction in roots but antagonistic interaction in coleoptiles was revealed between ABA and ethylene, providing new insights into understanding of their complicated interplay.

### Materials and Methods

#### Plant Materials and Growth Conditions

The *mhz4* and *Osein2/mhz7-1* mutants were identified previously [45]. The *OsERS1* (LOC_Os03g49500) T-DNA knockout mutant *Oser1* (PF418312.1) is in Dongjin ([2]) background and was obtained from the POSTECH Biotech Center [50]. The homogenous *Oser1* mutation was identified by PCR using the T-DNA left border primer PR152 (‘5’-TTGTTTA-GAGAACAGCACAA-3’) and gene-specific primers flanking the insertion site: PR139 (‘5’-AATAATGATGGCCAGAC-3’).
and PR140 (5′-TGCTTTCAGATCCTTTGGT-3′). The OsEIN2 (LOC_Os06g06130), and the mutation was identified as previously described [45]. For ethylene treatment, rice seedlings were grown on a stainless steel sieve that was placed in an air-tight plastic box with various concentrations of ethylene [45]. The seedlings were incubated at 28°C in the dark for 2 to 3 days as indicated in each experiment. For ABA or NDGA treatment, rice seedlings were grown on a stainless steel sieve and various concentrations of ABA (Sigma, A1049) or 100 μM NDGA (Sigma) was added to the water. The stock solutions of ABA and NDGA were prepared in ethanol. For AVG treatment, rice seedlings were grown on eight layers of cheesecloth saturated with 5 μM AVG (Sigma) in Petri dishes. For field experiments, rice plants were grown in the Experimental farm Station of the Institute of Genetics and Developmental Biology in Beijing from May to October of each year.

Map-Based Cloning of MHZ4 Gene

F2 mapping populations were generated from crosses between mhz4 mutant and indica variety Minghui 63. Genomic DNA was isolated from etiolated seedlings with mutant phenotype. For primary mapping, bulked segregant analysis (BSA) was performed using a DNA pool from 15 mutant individuals selected from the F2 population between mhz4 and MH63. The markers used in BSA included 30 published SSR markers [http://www.gramene.org] and 117 insertion-deletion (Idl) markers. A total of 480 mutant individuals selected from the F2 populations were used for fine mapping. MHZ4 locus was mapped to chromosome 1 between Idl1–1.55 and 5′-CAGGGCAATCTGTCAAAAGCT-3′ and 5′-CTAAAGATCAGTACTGGGCAC-3′ and Idl1–1.58 (5′-CTC-TTTGTCAAGCTTTATTTACC-3′) and 5′-ACAGATCCG-TATGTTTATAGT-3′) in the region of 1.55 Mb to 1.58 Mb (Genebank accession number: NC_008400), which contains 4 genes. The candidate gene was finally determined by DNA sequencing of all the candidate genes within this region.

To confirm the 18-bp deletion in the genomic DNA of mhz4, DNA fragment length polymorphism between wild-type (WT) and the mutant was examined using PCR with primers (5′-CTCTTGTCTCCGGCTCGGCA-3′ and 5′-AGGAGGGCGATGTTGGCCCA-3′). The 81-bp insertion in the mhz4 cDNA was detected by PCR amplification of the full-length cDNA using primers (5′-ATGGCCGATCTTCCTCTCTCT-3′ and 5′-TCAATGTGAGCGACCAATTGAAC-3′).

Quantitative PCR and RNA-Seq Analyses

Total RNAs were extracted from various rice tissues using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR and/or semi-quantitative PCR were carried out as described previously [45]. The primers are listed in Table S1. For RNA-seq analysis, two-day-old etiolated seedlings of WT and mhz4 mutant were treated with air or 10 ppm ethylene for 8 h. The libraries were prepared from 10 μg total RNA using NEBNext Ultra RNA Library Prep Kit (NEB, USA), and each sample contains two biological replicates. Clean reads were mapped to rice MSU7.0 genome using TopHat, and analyzed using Cufflinks as described [71].

Transient Expression Assay

To generate MHZ4-GFP fusion construct, the MHZ4 coding sequence was PCR amplified (for primers see Table S1), digested with XhoI/NcoI and fused in frame to the 5′-end of GFP in a pUC18-based vector under the control of CaMV35S promoter. The fusion gene was transiently expressed in tobacco leaves by microprojectile bombardment using a Bio-Rad PDS-1000/He particle delivery system [9]. The images were taken using a confocal microscope (Leica TCS SP5). Excitation/emission wavelengths were set at 488 nm/500–530 nm for GFP fluorescence and at 570 nm/640 nm for chlorophyll autofluorescence.

Generation of Transgenic Rice Plants

To generate MHZ4pro::GUS construct, a 2048-bp promoter region upstream of the start codon was PCR amplified and cloned into pSt83871/BamHI sites of pCAMBIA2300-35S-GUS vector to replace the CaMV35S promoter. To generate MHZ4-complementation construct, the genomic DNA (4213 bp) of MHZ4 was PCR amplified and subcloned into pSt83871/Sali-digested pCAMBIA2300-35S vector. To generate the 35S::MHZ4 construct, the MHZ4 coding sequence was PCR amplified and inserted into BamHI/SalI-digested pCAMBIA2300-35S vector. The primers used for these constructions are listed in Table S1. The constructs were transfected into Agrobacterium tumefaciens strain EHA105 by electroporation. Rice transformation was performed as previously described [42]. The MHZ4pro::GUS or 35S::MHZ4 construct was transformed into rice variety Nipponbare. The MHZ4-complementation construct was transferred into mhz4 mutant. Positive transgenic plants were confirmed by PCR using NPT II gene-specific primers (Table S1). Homozygous transgenic lines were selected using Kanamycin treatment (50 mg/L).

GUS Staining

Rice seedlings or organs were fixed in 90% acetone on ice for 15 min, rinsed with staining buffer (100 mM Na3PO4 buffer pH 7.0, 10 mM EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1% Triton X-100), and vacuum infiltrated with staining solution (staining buffer containing 0.5 mg/ml X-Gluc (Sigma, B8049) for 15 minutes. The samples were incubated at 37°C in the dark. After staining, green tissues were decolorized in 70% ethanol and then in ethanol/acetic acid (6:1) until the chlorophyll was removed. The samples were observed using stereo microscopy (Leica, M165 FC).

Measurement of Ethylene and ABA

Ethylene production of WT and mhz4 mutant in 4 d-old etiolated seedlings, two-week-old green seedlings, mature leaves and panicles was determined by gas chromatography (GC2014, Shimadzu, Japan) equipped with a flame ionization detector. ABA contents in WT and mhz4 were detected using 4 d-old etiolated seedlings or 2 d-old etiolated seedlings treated with or without 10 ppm of ethylene for 48 h. Plant tissues (200 mg) were ground into powder in liquid nitrogen. ABA was determined by UPLC-MS/MS (UPLC-Quattro Premier XE) at the National Center of Plant Gene Research (IGDB, CAS, Beijing, China) as described [72]. For both ethylene and ABA detection, each material or treatment includes three duplications.

Measurements of Agronomic Traits in Field-Grown Plants

Heading date of WT, mhz4 mutants and MHZ4-OX lines was recorded when the first spike of a rice plant emerged about 1 cm above the sheaths. Twenty to thirty individuals were examined for each material. After harvest, 20 plants from each material were used for measurements of agronomic traits as previously described [45].

Supporting Information

Figure S1 ABA dose-response curves for root and coleoptile growth in WT. Rice seedlings were grown in the dark for 3 d in...
the presence of various concentrations of ABA. Each point is average of 35 to 40 seedlings and bars indicate SD.

(TIF)

Figure S2 Effects of AVG treatment on ethylene production and coleoptile ethylene response of mhz4 and WT seedlings. (A) Ethylene production in the presence of various concentrations of AVG. Data are the mean ± SD of three replicates. (B) Coleoptile phenotypes of the seedlings grown in the dark for 2.5 days in the presence or absence of 10 ppm ethylene, supplemented with or without 5 μM AVG.

(TIF)

Figure S3 MHZ4 gene expression levels in overexpressing lines detected using semi-quantitative RT-PCR. Actin1 was used as a control.

(TIF)

Figure S4 Identification of the Oser1 mutant. (A) Schematic representation of OsERS1 [LOC_Os03g49500] gene structure. The T-DNA insertion site and the primer positions are indicated. (B) PCR genotyping for the Oser1 mutant and WT (Dongjin). (C) OsERS1 expression in Oser1 and WT detected by RT-PCR analysis with amplification of the full-length cDNA. Actin1 was used as an internal control.

(TIF)

Figure S5 Quantification of branching and adventitious rooting in WT, mhz4, Osen2 and mhz4 Osen2 double mutant plants in a different year. (A) Branch length at each node in main tillers from 20 plants. (B) Percentage of adventitious root formation at each node in main tillers in 20 plants in [A].

(TIF)

Figure S6 Preharvest sprouting and grain-related traits of mhz4 mutant. (A) Preharvest sprouting of mhz4. Arrow heads indicate germinated seeds on a panicle. (B) Germination rates of freshly harvested seeds of WT and mhz4. Each point is the percentage of germinated seeds among 100 seeds. (C) Comparison of grain thickness (top left panel), grain length (bottom panel) and their quantification results (top right panel) in WT and mhz4. Each column is an average of 100 grains and bars indicate SD. * and ** indicate significant difference compared to WT (P<0.01). (D) Time-course of grain-filling after fertilization. Each point is an average of 50–100 grains and bars indicate SD.

(TIF)

Table S1 Primers used gene expression analysis and plasmid construction.

(DOCX)

Table S2 Yield-related traits in WT, mhz4 mutant and MHZ4 overexpressing lines. Each value is average of 20 to 24 individuals. * and ** indicate significant difference compared to WT at P<0.05 and P<0.01, respectively. Data from two years are presented.

(DOCX)

Acknowledgments

We would like to thank Xiaohong Sun, Cunyu Yan and Jinfang Chu (National Centre for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) in determining the ABA contents.

Author Contributions

Conceived and designed the experiments: BM JSZ SYC. Performed the experiments: BM CCY SJH. Analyzed the data: BM XL. Contributed reagents/materials/analysis tools: TGL WKZ. Wrote the paper: BM JSZ.

References

1. Abeles FB, Morgan PW, Salveit JM (1992) Ethylene in Plant Biology. 2nd ed. (San Diego, CA: Academic Press).
2. Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18.
3. Hall B, Shakel S, Schaller GE (2007) Ethylene receptors: ethylene perception and signal transduction. J Plant Growth Regul 26: 110–130.
4. Ju C, Chang C (2012) Advances in ethylene signalling: protein complexes at the plasma membrane in Arabidopsis. Annu Rev Plant Biol 12: 479–485.
5. Kendrick MD, Chang C (2008) Ethylene signaling: new levels of complexity and regulation. Curr Opin Plant Biol 11: 479–485.
6. Chen YF, Randlett MD, Findell JL, Schaller GE (2002) Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of Arabidopsis. J Biol Chem 277: 19861–19866.
7. Gao ZY, Chen YF, Randlett MD, Zhao XG, Findell JL, et al (2003) Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complex. J Biol Chem 278: 34725–34732.
8. Ma B, Cui ML, Sun HJ, Takada K, Mori H, et al. (2006) Subcellular localization and membrane topology of the melon ethylene receptor CmERS1. Plant Physiol 141: 587–597.
9. Bisson MMA, Bleckmann A, Allekotte S, Groth G (2009) EIN2, the central regulator of ethylene signalling, is localized at the ER membrane where it interacts with the ethylene receptor ETR1. Biochem J 424: 1–6.
10. Gao H, Chang KN, Yazaki J, Ecker JR (2009) Interplay between ethylene, ETP1/ETP2, F-box proteins, and degradation of EIN2 triggers ethylene responses in Arabidopsis. Genes Dev 23: 512–521.
11. Ju C, Yoon GM, Shemansky JM, Lin DY, Ying ZI, et al. (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proc Natl Acad Sci USA 109: 19486–19491.
12. Kamiyoshihara Y, Tieman DM, Huber DJ, Klee HJ (2012) Ligand-induced alterations in the phosphorylation state of ethylene receptors in tomato fruit. Plant Physiol 160: 486–497.
13. Zhao Q, Guo H (2011) Paradigms and paradox in the ethylene signalling network. Trends Plant Sci 16: 1117–1126.
14. Gao ZY, Chen YF, Randlett MD, Zhao XG, Findell JL, et al. (2003) Subcellular localization and membrane topology of the melon ethylene receptor CmERS1. Plant Physiol 141: 587–597.
15. Bisson MMA, Bleckmann A, Allekotte S, Groth G (2009) EIN2, the central regulator of ethylene signalling, is localized at the ER membrane where it interacts with the ethylene receptor ETR1. Biochem J 424: 1–6.
16. Gao H, Chang KN, Yazaki J, Ecker JR (2009) Interplay between ethylene, ETP1/ETP2, F-box proteins, and degradation of EIN2 triggers ethylene responses in Arabidopsis. Genes Dev 23: 512–521.
17. Ju C, Yoon GM, Shemansky JM, Lin DY, Ying ZI, et al. (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proc Natl Acad Sci USA 109: 19486–19491.
18. Kamiyoshihara Y, Tieman DM, Huber DJ, Klee HJ (2012) Ligand-induced alterations in the phosphorylation state of ethylene receptors in tomato fruit. Plant Physiol 160: 486–497.
19. Zhao H, Shen Z, Huang S, Schmitz RJ, Urich MA, et al. (2012) Processing and subcellular trafficking of ER-released EIN2 control response to ethylene gas. Science 338: 390–393.
20. Wen X, Zhang C, Ji Y, Zhao Q, He W, et al. (2012) Acrivation of ethylene signaling is mediated by nuclear translocation of the cleared EIN2 carboxyl terminus. Plant Cell 22: 1613–1615.
21. Gao HW, Ecker JR (2003) Plant responses to ethylene gas are mediated by SGC (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell 115: 667–677.
22. Gao ZY, Chen YF, Randlett MD, Zhao XG, Findell JL, et al. (2003) Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complex. J Biol Chem 278: 34725–34732.
23. Benschop JJ, Millenaar FF, Smeets ME, van Zanten M, Voesenek LA, et al. (2005) ABA-regulated genes in ABA- and ethylene-dependent seedling growth. Plant Cell 17: 485–498.
24. Benschop JJ, Jackson MB, Guhl K, Vreeburg RAM, Croker SJ, et al. (2005) Contrasting interactions between ethylene and abscisic acid in Arabidopsis. Plant Cell 17: 26–38.
25. Wang Y, Liu C, Li K, Sun F, Hu H, et al. (2007) Arabidopsis F-box proteins: EBF1 and EBF2. Cell 115: 679–689.
26. Cheng WH, Chiang MH, Hwang SG, Lin PC (2009) Antagonism between abscisic acid and ethylene in Arabidopsis. Proc Natl Acad Sci USA 106: 1013–1018.
27. Beaudoin N, Serizet C, Gosti F, Giraudat J, Vrijsen N (2000) Interactions between abscisic acid and ethylene signaling cascades. Plant Cell 12: 1103–1115.
28. Ghassemian M, Nambara E, Cutler S, Kawade Y, Kamiya Y, et al. (2000) Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. Plant Cell 12: 1117–1126.
29. Benschop JJ, Jackson MB, Guhl K, Vreeburg RAM, Croker SJ, et al. (2005) Contrasting interactions between ethylene and abscisic acid in Rumex species differing in submergence tolerance. Plant J 44: 736–768.
30. Benschop JJ, Millenaar FF, Smeets ME, van Zanten M, Voesenek LA, et al. (2005) Arabidopsis F-box proteins: EBF1 and EBF2. Plant Cell 17: 485–498.
31. Vandenbusche F, Van Der Straeten D (2007) Overexpression of the melon ethylene receptor CmERS1. Plant Physiol 143: 1013–1023.
32. Gao ZY, Chen YF, Randlett MD, Zhao XG, Findell JL, et al. (2003) Subcellular localization and membrane topology of the melon ethylene receptor CmERS1. Plant Physiol 141: 587–597.
27. Dong H, Zhen Z, Peng J, Chang L, Gong Q, et al. (2011) Loss of ACS7 confers abiotic stress tolerance by modulating ABA sensitivity and accumulation in Arabidopsis. J Exp Bot 62: 4875–4887.

28. Li Z, Zhang L, Yu Y, Qian R, Zhang Z, et al. (2011) The ethylene response factor HY5 is a crucial repressor for ethylene biosynthesis in Arabidopsis. Plant J 68: 89–99.

29. Chen L, Dodd IC, Davies WJ, Wilkinson S (2013) Ethylene limits abscisic acid synthesis and induces stomatal closure in aged wheat leaves. Plant Cell Environ 36: 1850–1859.

30. Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56: 163–185.

31. Rock CD, Zeevaart JA (1991) The aba mutant of Arabidopsis confers both auxin and ethylene responsiveness. Plant Physiol 94: 662–666.

32. Dong H, Zhen Z, Peng J, Chang L, Gong Q, et al. (2011) Loss of

33. Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, et al. (2001) Regulation of the ethylene response pathway in Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci U S A 98: 7496–7499.

34. Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, et al. (2001) Regulation of the ethylene response pathway in Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci U S A 98: 7496–7499.

35. North HM, Almeida AD, Boutin JP, Frey A, To A, et al. (2007) The Arabidopsis ABA-deficient mutant aor4 demonstrates that the major route for stress-induced ABA accumulation is via neo-oxanthin isomers. Plant J 50: 810–824.

36. Neuman H, Galpaz N, Cunningham FX Jr, Zamir D, Hirshberg J (2014) The tomato mutation 

37. Rock CD, Zeevaart JA (1991) The aba mutant of Arabidopsis confers both auxin and ethylene responsiveness. Plant Physiol 94: 662–666.

38. Dong H, Zhen Z, Peng J, Chang L, Gong Q, et al. (2011) Loss of

39. Ben-Ari G (2012) The ABA signal transduction mechanism in commercial crops: various aspects of rice growth and development. J Exp Bot 64: 4863–4875.

40. Rock CD, Zeevaart JA (1991) The aba mutant of Arabidopsis confers both auxin and ethylene responsiveness. Plant Physiol 94: 662–666.

41. Rock CD, Zeevaart JA (1991) The aba mutant of Arabidopsis confers both auxin and ethylene responsiveness. Plant Physiol 94: 662–666.

42. Dong H, Zhen Z, Peng J, Chang L, Gong Q, et al. (2011) Loss of

43. Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, et al. (2001) Regulation of the ethylene response pathway in Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci U S A 98: 7496–7499.

44. Wang Q, Zhang W, Yin Z, Wen CK (2013) Rice CONSTITUTIVE TRIPLE RESPONSE (OsCPR1) mediates auxin responsiveness and enhances flowering and yield trait regulation. Mol Plant 6: 1830–1848.

45. Wang Q, Zhang W, Yin Z, Wen CK (2013) Rice CONSTITUTIVE TRIPLE RESPONSE (OsCPR1) mediates auxin responsiveness and enhances flowering and yield trait regulation. Mol Plant 6: 1830–1848.

46. Ku HS, Suge H, Rappaport L, Pratt HK (1970) Stimulation of rice coleoptile photo-oxidation in rice. Plant J 50: 810–824.

47. Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, et al. (2009) Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol Plant 2: 1134–1140.

48. Lenka SK, Lohia B, Kumar A, Chinnusamy V, Bansal KC (2009) Genome-wide targeted prediction of ABA responsive genes in rice based on co-expressed genes. Plant Mol Biol 69: 261–271.

49. Saika H, Okamoto M, Mikiyo K, Kushiro T, Shinoda S, et al. (2007) Ethylene promotes submergence-induced expression of OsABA8ox1, a gene that encodes ABA β'-hydroxylase in rice. Plant Cell Physiol 48: 287–298.

50. Yi J, An G (2013) Utilization of T-DNA tagging lines in rice. J Plant Biol 56: 85–90.

51. Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie D, et al. (2008) T/AIL-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 133:177–191.

52. Perrine Z, Negi S, Richard T, Sayre RT (2012) Optimization of photosynthetic light energy utilization by microalgae. Algal Res 1: 134–142.

53. Ma B, Chen SY, Zhang JS (2010) Ethylene signaling in rice. Chinese Sci Bull 55: 2204–2210.

54. Fang J, Chai C, Qian Q, Li C, Tang J, et al. (2008) Mutations of genes in the synthesis of the central precursors of ABA lead to pre-harvest sprouting and photo-oxidation in rice. Plant J 54: 177–189.

55. Rock CD, Zeevaart JA (1991) The aba mutant of Arabidopsis confers both auxin and ethylene responsiveness. Plant Physiol 94: 662–666.

56. Luscha C, Guadella RA, Grisoli P, Fink GR (1990) ERT1, a root-specific protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana. Genes Dev 12: 2175–2187.

57. Alonso JM, Stepanova AN, Solano R, Wissman E, Ferrari S, et al. (2005) Five component of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc Natl Acad Sci U S A 102: 2992–2997.

58. Kissick K, Ljung K, Vanneste S, Podloska R, Beckman T, et al. (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell 19: 2197–2212.

59. Stepanova AN, Hoyt JM, Hamilton AA, Alonso JM (2005) A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in Arabidopsis. Plant Cell 17: 2230–2242.

60. Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie D, et al. (2008) T/AIL-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 133:177–191.

61. Stepanova AN, Hoyt JM, Hamilton AA, Alonso JM (2005) A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in Arabidopsis. Plant Cell 17: 2230–2242.

62. Swarup R, Perry P, Hagenbeck D, Van Der Straaten D, Beemster GT, et al. (2007) Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. Plant Cell 19: 2186–2196.

63. Wang Q, Zhang W, Yin Z, Wen CK (2013) Rice CONSTITUTIVE TRIPLE RESPONSE is involved in the ethylene-receptor signalling and regulation of various aspects of rice growth and development. J Exp Bot 64: 4875–4877.

64. Ma B, He SJ, Duan KX, Yin CC, Chen H, et al. (2013) Identification of rice ethylene-response mutants and characterization of MEH2/OE2IN2 in distinct ethylene response and yield trait regulation. Mol Plant 6: 1030–1046.

65. Wang Q, Zhang W, Yin Z, Wen CK (2013) Rice CONSTITUTIVE TRIPLE RESPONSE is involved in the ethylene-receptor signalling and regulation of various aspects of rice growth and development. J Exp Bot 64: 4875–4877.

66. Hoffmann-Benning S, Benfow K (2012) Hormonal interactions in the regulation of plant development. Annu Rev Cell Dev Biol 28: 22.1–22.25.

67. Leopold ME, Spollen WG, Sharp RE (2004) Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. J Exp Bot 55: 237–245.

68. Hoffmann-Benning S, Kende H (1992) On the role of abscisic acid and gibberellin in the regulation of growth in rice. Plant Physiol 99: 1156–1161.

69. Leopold ME, Spollen WG, Sharp RE (2004) Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. J Exp Bot 55: 237–245.

70. Sharp RE, Leopold ME, Else MA, Thorne ET, Gherardi F (2009) Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: evidence for an interaction with ethylene. J Exp Bot 51: 1575–1584.

71. Trappnell C, Roberts A, Geoff L, Preece G, Kini D, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protocols 7: 562–578.

72. Fu J, Chiu J, Sun X, Wang J, Yan C (2012) Simple, rapid, and simultaneous assay of multiple carbonyl containing phytohormones in wounded tomatoes by UPLC-MS/MS using single SPE purification and isotope dilution. Anal Sci 28: 1081–1087.