Histidine kinase MHZ1/OsHK1 interacts with ethylene receptors to regulate root growth in rice

He Zhao, Kai-Xuan Duan, Biao Ma, Cui-Cui Yin, Yang Hu, Jian-Jun Tao, Yi-Hua Huang, Wu-Qiang Cao, Hui Chen, Chao Yang, Zhi-Guo Zhang, Si-Jie He, Wan-Ke Zhang, Xiang-Yuan Wan, Tie-Gang Lu, Shou-Yi Chen & Jin-Song Zhang

Ethylene plays essential roles during adaptive responses to water-saturating environments in rice, but knowledge of its signaling mechanism remains limited. Here, through an analysis of a rice ethylene-response mutant mhz1, we show that MHZ1 positively modulates root ethylene responses. MHZ1 encodes the rice histidine kinase OsHK1. MHZ1/OsHK1 is autophosphorylated at a conserved histidine residue and can transfer the phosphoryl signal to the response regulator OsRR21 via the phosphotransfer proteins OsAHP1/2. This phosphorelay pathway is required for root ethylene responses. Ethylene receptor OsERS2, via its GAF domain, physically interacts with MHZ1/OsHK1 and inhibits its kinase activity. Genetic analyses suggest that MHZ1/OsHK1 acts at the level of ethylene perception and works together with the OsEIN2-mediated pathway to regulate root growth. Our results suggest that MHZ1/OsHK1 mediates the ethylene response partially independently of OsEIN2, and is directly inhibited by ethylene receptors, thus revealing mechanistic details of ethylene signaling for root growth regulation.
Ethylene plays essential roles in plant growth and development. In the model plant Arabidopsis, a linear ethylene signaling pathway has been established based on the characterization of a series of triple response mutants1-4. The pathway contains a family of endoplasmic reticulum (ER) membrane-bound ethylene receptors, a Ser/Thr kinase CTR1 (constitutive triple response 1), a central ER membrane protein EIN2 and transcription factors EIN3/EIL1 and ERF1-13.

In the absence of ethylene, the ethylene receptors are in active state and CTR1 is likely activated to phosphorylate the C-terminal domain of EIN2 to repress ethylene response. In the presence of ethylene, the C-terminus of EIN2 is cleaved and translocated to nucleus for activation of downstream EIN3/EIL1 transcriptional cascade and then ethylene response14-16. EIN2 and EIN3/EIL1 are regulated by proteasomal degradation17-20. Recently, the EIN2 C-terminal domain is also found to be targeted to the cytoplasmic processing-body (P-body) for the cytoplasmic processing-body (P-body) for the cytoplasmic processing-body (P-body) for the cytoplasmic processing-body (P-body).

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Rice (Oryza sativa) is an important monocotyledonous crop and usually lives in water-saturated soil in most of the life cycle. Despite the essential role ethylene plays in the adaptive responses of rice to hypoxia stress, the ethylene signaling pathway in rice has not been systematically studied. Genes homologous to the Arabidopsis ethylene signaling components have been identified and some are characterized in rice, including ethylene receptor gene OsETR2, RTE1-like gene, CTR1-like gene, EIN2-like gene and EIN3-like gene26-30. Adopting an effective screening system, we have isolated a set of rice ethylene-response mutants31. The analyses of these mutants suggest that ethylene signaling in rice and Arabidopsis has both conserved and divergent aspects32-35.

Histidine kinases (HK) play crucial roles in the regulation of plant development in response to hormones, as well as environmental stimuli36,37. HK-mediated multistep phosphorylase involves hybrid-type HK with both histidine kinase and receiver domains, His-containing phosphotransfer protein (HPT1), and response regulator (RR)37,38. Ethylene receptors are structurally similar to bacterial HKs and some receptors such as ETR1 and ERS1 do have canonical HK activity39,40. However, the HK activity of ethylene receptor is not required for ethylene signaling but only plays a modulating role in the pathway41,42. So far, how the ethylene receptor transmits signals remains largely unclear. It has been reported that a non-ethylene receptor HK, Arabidopsis authentic HK5 (AHK5), acts as a negative regulator in the ETR1 dependent signaling pathway in which ethylene and ABA inhibit the root elongation43. In contrast, the maize homolog ZmHK9 acts as a positive regulator in the root growth response to ethylene and ABA in transgenic Arabidopsis44. OsHK1, a rice histidine kinase38, is reported to play roles in root growth and circummutations through a cytokinin-related pathway45. In these studies, however, little is known about the molecular mechanism by which the HKs regulate the signaling cascade.

In this study, we characterized the rice root-specific ethylene-insensitive mutant mhz1 and found that MHZ1 encodes the rice histidine kinase OsHK1. MHZ1 positively modulates ethylene response in rice roots. Biochemical analysis showed that MHZ1 is a functional hybrid-type HK, which autophosphorylates in a conserved histidine and transfers the phosphoryl group via its receiver domain to OsAHP1/2 and then further to response regulator OsRR21. Genetic evidence demonstrates that the HK activity of MHZ1 and it-mediated phosphorylation are required for regulation of root ethylene response in rice. More interestingly, we discover that the ethylene receptors, via GAF domain, can directly bind to MHZ1 protein and inhibit its kinase activity based on both in vitro and in vivo analyses. These findings reveal a previously unidentified mechanism for the ethylene receptor signal transduction.

Results
Characterization of mhz1 and gene identification. We have isolated a set of rice ethylene-response mutants and the mhz1 exhibited root-specific ethylene-insensitive phenotype31. In air, etiolated seedlings of two allelic mutants mhz1-1 and mhz1-2 were very similar in coleoptile/shoot and root growth to WT. In ethylene, WT root length was drastically reduced whereas mhz1-1 and mhz1-2 root growth was not inhibited, indicating a complete ethylene-insensitive phenotype in primary roots of the two mutants (Fig. 1a). Coleoptile growth of mhz1-1 and mhz1-2 responded normally to ethylene, except that the mutants have slightly longer coleoptiles than WT (Fig. 1a). Light-grown mhz1-1 seedlings had longer roots than WT (Supplementary Fig. 1a). Two additional allelic mutants (mhz1-3 and mhz1-4) were further identified and they resembled mhz1-1 and mhz1-2 in ethylene responses (Supplementary Fig. 1b). These results indicate that mhz1 is insensitive to ethylene in root growth.

The MHZ1 gene was identified to be LOC_Os06g44410 through TAIL-PCR analysis and the T-DNA was inserted in the fifth intron between 2032 bp and 2033 bp from the start codon of the gene in mhz1-1 (Fig. 1b, c). No MHZ1 expression was detected in mhz1-1 (Fig. 1d). Other alleles were further analyzed (Fig. 1b, c and Supplementary Fig. 1b). Genetic transformation with the WT genomic DNA fragment rescued the ethylene-insensitive phenotype of mhz1-1 (Supplementary Fig. 1c). All these results indicate that MHZ1 corresponds to the locus LOC_Os06g44410, which encodes the histidine kinase OsHK138. The mhz1-1, -2, -3, -4, -5 mutants may be renamed as OsHK1-4, -5, -6, -7, -8 following the OsHK1 mutants identified by Lehner et al.45. For simplicity, the original mutant names were used since these mutants have been named as mao huizi (mhz, Chinese name with an English meaning of cat whiskers)31.

MHZ1 overexpression enhances ethylene response in roots. To study the gene function of MHZ1, we overexpressed MHZ1 in WT rice (Fig. 1e, f and Supplementary Fig. 4d). Compared with the WT, the high-expression MHZ1-OE lines had shorter roots both in air and in ethylene, indicating a constitutive ethylene-response phenotype (Fig. 1e). The short root phenotype of MHZ1-OE lines was not due to elevation of ethylene emission through TAIL-PCR analysis and the T-DNA was inserted in the fifth intron between 2032 bp and 2033 bp from the start codon of the gene in mhz1-1 (Fig. 1b, c). No MHZ1 expression was detected in mhz1-1 (Fig. 1d). Other alleles were further analyzed (Fig. 1b, c and Supplementary Fig. 1b). Genetic transformation with the WT genomic DNA fragment rescued the ethylene-insensitive phenotype of mhz1-1 (Supplementary Fig. 1c). All these results indicate that MHZ1 corresponds to the locus LOC_Os06g44410, which encodes the histidine kinase OsHK138. The mhz1-1, -2, -3, -4, -5 mutants may be renamed as OsHK1-4, -5, -6, -7, -8 following the OsHK1 mutants identified by Lehner et al.45. For simplicity, the original mutant names were used since these mutants have been named as mao huizi (mhz, Chinese name with an English meaning of cat whiskers)31.

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We further examined expression of ethylene-responsive genes identified in our previous studies31,32,34,35. qPCR analysis showed that ethylene-induction of OsRRA5, OsERF002 and OsRAP2.8 expression was largely blocked in mhz1, whereas the expression of these genes was substantially enhanced in roots of
MHZ1-overexpressing plants in the presence or absence of ethylene (Fig. 1f). In shoots of MHZ1-overexpressing plants, OsRRA5 and OsRAP2.8 expression was not enhanced in air or ethylene; whereas OsERF002 expression was promoted compared to the corresponding WT levels especially in the presence of ethylene (Fig. 1f). The ethylene induction of OsERF063 and OsERF073 was not affected in both roots and shoots of mhz1 or MHZ1-OE plants, suggesting that expression of some genes is independent of MHZ1 (Fig. 1f). These results further confirmed the different ethylene responsiveness of mhz1 mutant and MHZ1-OE plants at molecular levels with some organ and gene specificity.
Fig. 1 MHZ1 positively regulates the ethylene response in rice roots. a Ethylene response phenotype of mhz1 alleles. Etiolated seedlings were treated with various concentrations of ethylene in darkness. Representative seedlings grown in the air and in 10 ppm ethylene are shown (Left). Coleoptile (Center) and root lengths (Right) are means ± SD, n > 30. Bars indicate 10 mm. b MHZ1 genomic structure and mutation sites of different mhz1 alleles. Colored boxes indicate exons and horizontal lines indicate introns. c Schematic structure of MHZ1 and mutation sites of different mhz1 alleles. d MHZ1 gene expression in WT and mhz1-1. Actin1 was amplified as internal control. e MHZ1 overexpression lines (MHZ1-OE) have constitutive ethylene response. MHZ1 native promoter was used to drive the MHZ1 cDNA for overexpression. Etiolated seedlings were treated with various concentrations of ethylene and 10 ppm 1-MCP under darkness. Bars indicate 10 mm. Root lengths (Right) are means ± SD, n > 30. f Expression of ethylene-inducible genes OsRRAS, OsERF002, OsRAP2.8, OsERF063 and OsERF073 in mhz1-1 and MHZ1-OE lines compared with WT as revealed by qPCR. Data are means ± SD, n = 4. Source data are provided as a Source Data file.

Ethylene-induced MHZ1 expression requires OsEIN2 and OsEIL1. MHZ1 transcripts are abundant in roots but less in coleoptiles and other organs, and are induced by ethylene in roots (Fig. 2a and Supplementary Fig. 4a). The ethylene induction of MHZ1 requires OsEIN2 and OsEIL1, and OsEIL1 can bind to the ATGTA elements in the MHZ1 promoter and activate the promoter activity in a tobacco transient assay system (Fig. 2a, b, c). Promoter-GUS analysis further reveals that MHZ1 promoter activity is mainly localized in root initiation sites at node, root vascular cylinder and root cortex. The activity is also observed in stem, leaf, grain hull and coleoptile (Supplementary Fig. 4b). Ethylene treatment mildly enhanced the MHZ1 promoter activity especially in the region immediately above the meristem tissue of root tip (Supplementary Fig. 4c).

MHZ1 transfers phosphoryl groups to OsRR21 via OsAHPs. Next, we examined whether MHZ1 has HK activity. Different mutant proteins or truncated versions were produced and tested for autophosphorylation ability (Fig. 3a). The GST-MHZ1 protein (amino acids 365 to 968) containing the kinase domain and receiver domain displays strong kinase activity in the presence of Ca2+ in our phosphorylation assay, and this activity was abolished when the conserved His at 375 position was mutated to Gln (Fig. 3b). A smaller radioactive band was noted below the normal GST-MHZ1, likely representing a degradation product (Fig. 3b). At the physiological level of ATP concentration, GST-MHZ1 had kinase activity in the presence of Ca2+ or Mg2+ (Supplementary Fig. 6a). These results indicate that MHZ1 is a functional HK.

When the receiver domain was removed, GST-KD or MBP-KD containing kinase domain (amino acids 365 to 655) still had autophosphorylation activity (Fig. 3c). When the conserved G1 or G2 box for ATP binding in the kinase domain was mutated in MBP-G1 (G588A, G590A) or MBP-G2 (G618A, G620A), the kinase activity was disrupted (Fig. 3c). However, the two mutated proteins can be phosphorylated by the normal kinase domain GST-KD (Fig. 3c), suggesting that MHZ1 phosphorylation can occur in trans manner.

Next, we investigated whether MHZ1 can transfer phosphoryl groups to putative downstream HPt and B-type RR components in rice. Rice has five HPt (OsAHP1/2, OsPHH1/2/3) and more B-type RR47,48. Results showed that GST-MHZ1 can transfer phosphoryl groups to OsAHP1 and OsAHP2 instead of OsPHP1 or OsPHP2 (Fig. 3d), possibly implying substrate specificity. The OsPHP3 was not tested because OsPHP3 is a more diverged pseudogene47. H79Q and H80Q mutations in OsAHP1 and OsAHP2, respectively, disrupted their phosphorylation by GST-MHZ1 (Fig. 3e), suggesting that these residues are likely to be the phosphoryl-accepting sites.

Among the rice B-type response regulators, we selected the originally identified six (OsRR21 to OsRR26)47 to express in E. coli. Only OsRR21 and OsRR26 were successfully expressed, and
the OsRR21 but not OsRR26 can accept the phosphoryl group transferred from the phosphorylated OsAHP1 or OsAHP2 (Fig. 3f, g), suggesting substrate specificity. D68E mutation of OsRR21 abolished its phosphorylation (Fig. 3f, right panel), suggesting that D68 may be the phosphoryl-accepting site. D824A mutation in the receiver domain of GST-MHZ1 disabled the phosphorelay from MHZ1 to OsAHP1 or OsAHP2 (Fig. 3g, the left two lanes), indicating that the D824 residue of MHZ1 is indispensable for phosphotransfer from GST-MHZ1 to OsAHP1 or OsAHP2. All these results support that MHZ1 can autophosphorylate and transfer the phosphoryl group via its receiver domain to OsAHP1/OsAHP2 and then further to OsRR21 through phosphorelay.

Time-course analysis of the phosphorelay between GST-MHZ1, OsAHP1/2 and OsRR21 was performed to verify the MHZ1-mediated phosphorelay system. GST-MHZ1 was autophosphorylated and then incubated with OsAHP1. Over the time course, GST-MHZ1 phosphorylation was steadily enhanced (Fig. 3h, left panel).
Fig. 3 MHZ1-mediated phosphorelay pathway is required for ethylene-inhibited root growth. a MHZ1 and its truncated/mutant versions used for phosphorylation analysis. H, G1, G2, and D indicate conserved residues or boxes. MBP indicates maltose-binding protein, b MHZ1 has histidine kinase activity. The radioactive band below the GST-MHZ1 indicates a degradation product. c Trans-phosphorylation between MHZ1 molecules. MBP-G1 has G588A and G590A mutations at G1 box. MBP-G2 has G618A and G620A mutations at G2 box. d MHZ1 can transfer its phosphoryl groups to OsAHP1 and OsAHP2 rather than OsPHP1 or OsPHP2. e Phosphorelay from MHZ1 to OsAHP1 and OsAHP2 was abolished when the conserved histidine was mutated in OsAHP1/2. f MHZ1 can transfer its phosphoryl groups to OsAHP1/2 and further to OsRR21. D824E mutation in OsRR21 disrupted its phosphoryl-accepting ability. g D824A mutation in MHZ1 receiver domain blocked its phosphorelay to OsAHP1/2, and OsRR26 cannot accept phosphoryl groups transferred from MHZ1 and OsAHP1/2. h Time course of the phosphorelay from MHZ1 to OsAHP1 (left panel) and further to OsRR21 (right panel). After the reaction in the left panel was finished, the OsRR21 was added and incubated for various times in the right panel. i MHZ1 but not its mutant versions rescued the ethylene-insensitive phenotype of mh1 (mh1-1) mutant. cDNAs of MHZ1 and its mutant versions MHZ1(G1), MHZ1(H375Q) and MHZ1(D824A), fused with a 3 × FLAG sequence, driven by MHZ1 native promoter, were transformed into the mh1-1 to observe the root ethylene response. Total proteins of each line were immunoblotted for MHZ1-FLAG with anti-FLAG antibody. A non-specific band was used as a loading control. j MHZ1 gene expression was examined by RT-PCR and Actin1 was amplified as a control. Bar indicates 10 mm. k Ethylene response of Osahp1 Osahp2 double-mutant. Osahp1 Osahp2 double-mutant was segregated from the self-bred progenies of an Osahp1 (heterozygous)/Osahp2 (homozygous) plant. “+” indicates wild-type Osahp1. l Ethylene response of OsRR21 mutants and overexpression lines (OE). For f and k, etiolated seedlings were treated with 10 ppm ethylene or air for 2.5 days. Bars indicate 10 mm. Source data are provided as a Source Data file.

panel), suggesting an active phosphotransfer from GST-MHZ1 to OsAHP1. After phosphotransfer from GST-MHZ1 to OsAHP1, the OsRR21 was further added to the assay system and its phosphorylation level was also increased (Fig. 3h, right panel). By contrast, as negative controls, GST-MHZ1 plus OsSRR21, or OsAHP1 plus OsSRR21 did not result in OsSRR21 phosphorylation (Fig. 3h, right panel). Similar phosphotransfer also happened from GST-MHZ1 to OsAHP2 and further to OsSRR21 (Supplementary Fig. 6b). Altogether, all these data indicate that the activated GST-MHZ1 could transfer its phosphoryl group to OsAHP1/OsAHP2 and further to the downstream RRs, e.g., OsSRR21.

Ethylene signaling requires MHZ1-mediated phosphorelay. We further analyzed whether MHZ1 kinase activity and its-mediated phosphorelay is essential for ethylene response in rice roots. The coding region of MHZ1 gene tagged with FLAG and driven by MHZ1 native promoter, was mutated in the G1 box (G588A, G590A), the H375 (H375Q) or the D824 (D824A) sites and transformed into mh1-1. No ethylene response was observed in the roots of homozygous transgenic lines harboring the mutated MHZ1 genes, although the MHZ1-FLAG protein was detected in each homozygous line (Fig. 3i). As a positive control, the roots of mhz1-1 single mutants harbored the same ethylene response phenotype of the receptor mutants. Next, we used a dominant gain-of-function mutant Osers2d to test its genetic interaction with MHZ1. Osers2d was identified as mh1z2 from our ethyl methanesulphonate (EMS)-mutagenized population and harbors a dominant mutation (A32V) at the transmembrane domain of OsERS2, which is equivalent to Arabidopsis etr1-3 (Supplementary Fig. 8). The mutant showed ethylene-insensitive phenotype (Fig. 4b and Supplementary Fig. 8). The Osers2 mutation was introduced into MHZ1 overexpression background by crossing and by transgenic methods, without altering the abundance of MHZ1 protein (Fig. 4b, c). These results indicate that gain-of-function mutation of Osers2d suppressed MHZ1 function in root ethylene response. Combining with the fact that mh1z2 suppressed the short root phenotype of Osers2, we propose that MHZ1 may genetically function at the OsERS2 level or they may form a complex.

MHZ1 genetically acts at OsERS2. Genetic analyses were performed to study how MHZ1 interacts with the canonical ethylene signaling pathway. Double-mutant analysis showed that the ethylene hypersensitivity in the roots of Osers2 and Osers2 ethylene receptor loss-of-function mutants was abolished by mh1z2 mutation, suggesting that MHZ1 is required for the ethylene-response phenotype of the receptor mutants.
sugest that MHZ1 interacts with OsERS2 in yeast cells (Fig. 4e). GST pull-down assay and coimmunoprecipitation (Co-IP) assay in rice protoplasts further reveals that OsERS2 can interact with MHZ1 both in vitro and in rice cells (Fig. 4f, g). Since MHZ1 lacked the transmembrane domain and is predicted to be a cytoplasmic protein, we examined whether its interaction with OsERS2 could help it localize to the ER membrane through the membrane recruitment assay (MeRA)30. MHZ1-GFP and OsERS2-mCherry proteins were transiently expressed in tobacco leaf epidermal cells and fluorescence was examined. When expressed separately, OsERS2-mCherry was mainly detected in a reticular network-like structure, while MHZ1-GFP was mainly detected in the cytoplasm. When expressed together, MHZ1-GFP was found to co-localize with OsERS2-mCherry, suggesting that OsERS2 facilitated the ER membrane localization of MHZ1 (Fig. 4h). In addition, protein fractionation assay shows that a small amount of MHZ1 protein was detected in the membrane fractions even in the presence of gain-of-function Osers2d,
further supporting the association of MHZ1 with membrane-bound OsERS2 (Supplementary Fig. 9a). Consistently, 1-MCP treatment caused abundance of MHZ1 in membrane fraction, whereas ethylene treatment led to decrease of MHZ1 in membrane fraction (Supplementary Fig. 9b). Furthermore, Osers2d appeared to have stronger interaction with MHZ1 than wild-type OsERS2 in yeast two-hybrid assay (Supplementary Fig. 9c). To examine which domain of OsERS2 mediates the interaction with MHZ1, we generated truncated versions of OsERS2 (Fig. 4d). Co-IP assay shows that the GAF domain, but not the kinase domain (KD) of OsERS2, is actually responsible for the interaction of OsERS2 with MHZ1 (Fig. 4i).

Next, we examined the effects of this interaction on MHZ1 activity. In the phosphorylation assay, addition of increasing amount of OsERS2 (GST-ERS2ΔTM) drastically reduced the MHZ1 histidine kinase activity, whereas inclusion of GST itself did not significantly affect this activity (Fig. 4i), indicating that OsERS2 can inhibit the autophosphorylation of MHZ1 in vitro. Since autophosphorylated MHZ1 can transfer its phosphoryl group to OsAHPs (Fig. 3), we investigated whether OsERS2 could affect this process. Compared with GST, addition of the OsERS2 apparently reduced the OsAHP1 phosphorylation by MHZ1-mediated phosphorelay (Fig. 4k). The inhibitory effect of OsERS2 on MHZ1 kinase activity and the phosphorelay may not be due to an competitive binding of [γ-32P]ATP, as suggested by our results that OsERS2 only had very limited kinase activity in the presence of Ca2+ (Fig. 4k, right-most panel, Supplementary Fig. 10a). Given that the GAF domain mediates the interaction of OsERS2 with MHZ1, we examined the effect of GAF domain on MHZ1 autophosphorylation. Phosphorylation assay showed that the GAF domain exerted an inhibitory effect on MHZ1 autophosphorylation while the kinase domain of OsERS2 (GST-ERS2KD) did not show a significant effect (Fig. 4l). All these results indicate that OsERS2 can inhibit both MHZ1 autophosphorylation and MHZ1-mediated phosphorelay likely via its GAF domain.

To examine whether OsERS2 inhibits MHZ1 phosphorylation in vivo, we transfected vectors harboring the OsERS2-myc and Osers2d-myc into rice protoplasts isolated from mhz1/OsEIN2 plants (Fig. 9d). Rice protoplasts further showed that compared with total ERGs, MHZ1-dependent ERGs (Fig. 5f and Supplementary Data 1), we further compared the ethylene responsiveness of mhz1 and Osein2 by checking the expression of several ERGs, including OsRRA5, OsRAP2.8, OsERF002, OsERF063, and OsERF073. Whereas the ethylene induction of all five genes were abolished or hampered in Osein2, only OsRRA5, OsRAP2.8, and OsERF002 expression was affected by mhz1 (Supplementary Fig. 11a, b), suggesting that the ethylene responsiveness of mhz1 and Osein2 is differential in terms of gene expression. These results suggest that MHZ1-mediated pathway shares a subset of ERGs with the OsEIN2 signaling pathway for regulating root growth in rice, although genetically MHZ1 is partially independent of OsEIN2. A GO analysis for the subset of MHZ1-dependent ERGs in comparison to total ERGs was performed using BiNGO. Results showed that compared with total ERGs, MHZ1-dependent ERGs are mainly enriched in auxin signaling pathway and responses to different stimuli (Supplementary Fig. 11c). This is in line with former findings that ethylene functions upstream of auxin signaling to regulate root growth, indicating that MHZ1 may be involved in the crosstalk between ethylene, auxin and different stimuli to regulate root growth.

During an effort to screen for the suppressor of OsEIN2-Overexpression plants, three suppressor lines (SOE-7407, -410, -9744) were found to be very similar to the mhz1/OsEIN2-Overexpression seedlings after ethylene treatment (Supplementary Fig. 12). MHZ1 was identified to harbor the mutation sites in these suppressors by sequencing (Supplementary Fig. 12). These findings further support the genetic relationship between MHZ1 and OsEIN2.

### Discussion

Through mutant analysis, we identified MHZ1 as a positive modulator of root ethylene response in rice. MHZ1 has autophosphorylation ability and can transfer its phosphoryl group to OsRR21 via OsAHP1 and OsAHP2. Ethylene receptor OsERS2 physically interacts with MHZ1 to inhibit MHZ1 autophosphorylation and signaling. We propose that in the absence of ethylene, the ethylene receptors are in active conformations, which facilitates their interaction with MHZ1 and MHZ1 kinase

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**Genetic interaction of MHZ1 with OsEIN2.** We further examined the genetic relation of MHZ1 with OsEIN2. Rice Osein2 mutant is insensitive to ethylene in both root and coleoptile growth, and overexpression of OsEIN2 in WT seedlings resulted in strong constitutive and enhanced ethylene responses. We generate the mhz1/OsEIN2-Overexpression plants by crossing. OsEIN2-Overexpression partially suppressed the ethylene-insensitive root growth of mhz1 in mhz1/OsEIN2-Overexpression seedlings in ethylene (Fig. 5a, b), while the mhz1/OsEIN2-Overexpression seedlings still have longer adventitious roots at the node above the mesocotyl compared to the OsEIN2-Overexpression seedlings, indicating that activated OsEIN2-mediated signaling pathway can partially restore the ethylene response of mhz1 mutant (Fig. 5c).

We further crossed MHZ1-Overexpression with Osein2 to generate Osein2/MHZ1-Overexpression plants (Fig. 5d). In ethylene, the roots of Osein2/MHZ1-Overexpression seedlings are longer than that of MHZ1-Overexpression seedlings, but are still significantly shorter compared with its air control (Fig. 5d, e). The results indicate that Osein2 mutation cannot completely block the enhanced ethylene response conferred by MHZ1 overexpression, implying that MHZ1 may have the ability to accept signal from upstream components, e.g., ethylene receptors, independent of OsEIN2 function.
activity is suppressed. Upon ethylene perception, the receptors may release their inhibition on MHZ1, triggering MHZ1-mediated phosphorelay for regulation of root growth. This pathway may be a branch in parallel with the OsEIN2-mediated one, and both act downstream of ethylene receptor signaling to regulate root growth in rice (Fig. 5g).

Our study reveals a previously unidentified mechanism, by which ethylene receptor OsERS2 binds to the histidine kinase MHZ1 and suppresses its autophosphorylation and phosphorelay, adding knowledge toward how the ethylene receptors transmit signals. This conclusion may be in line with those from previous studies. In Arabidopsis, including ethylene receptors, OsCTR2, OsEIN2, and OsEIL1. Besides the conserved receptor-CTR2-OsEIN2-OsEIL1 signaling pathway, our results suggest a novel MHZ1-AHP1/2-OsRR21 phosphorelay pathway, through which ethylene receptors could regulate the root growth of rice by suppressing the kinase activity of MHZ1. In the absence of ethylene, ethylene receptors are in active conformations, which may facilitate their interaction with MHZ1 and suppress MHZ1 activity. With ethylene, ethylene receptors possibly released the repression effect on MHZ1 and the phosphorelay pathway is activated. The MHZ1-mediated phosphorelay pathway and the OsEIN2-mediated phosphorelay path may work together to regulate a subset of downstream gene to modulate root growth. MHZ1 and OsRRs can also be transcriptionally induced by ethylene through the OsEIN2-regulated phosphorelay pathway, which may facilitate the maintenance of MHZ1-mediated signaling after activation. The orange arrow indicates transcriptional activation. In addition, H₂O₂ may also function through MHZ1 to participate in the ethylene-regulated root growth. Source data are provided as a Source Data file.

**Fig. 5 Genetic interaction of MHZ1 and OsEIN2-mediated pathways.** a Ethylene response of mhz1/OsEIN2-OE in comparison with mhz1 and OsEIN2-OE. Bars indicate 10 mm. b Quantification of root length of the mutants in a. Root lengths are means ± SD, n > 30. **P < 0.01; Student’s t-test. c Enlargement of root features of the mutants treated with 10 ppm ethylene in a. d Ethylene response of OsEIN2/MHZ1-OE in comparison with OsEIN2 and MHZ1-OE. Rice seedlings were grown in dark for 2 days with air or 10 ppm ethylene. Bars indicate 10 mm. e Quantification of root length (above) and relative root length (below) of the mutants in d. Data are means ± SD, n > 30. **P < 0.01; Student’s t-test. f Comparison of MHZ1-, OsEIN2-, and OsEIL1-regulated ethylene-response genes. Etiolated seedlings of WT, mhz1, OsEIN2 and OsEIL1 were grown for 2 days before treated with air or 10 ppm ethylene for 3 h. Roots were subjected to RNA-seq analysis with three biological replicates. Ethylene-response genes (ERGs) were identified in WT according to the gene expression levels with at least relative twofold changes (q-value < 0.05) in ethylene treatment compared to those in the air. A total of 1789 ERGs were identified. g A proposed working model for MHZ1-regulated ethylene signaling in rice. Rice has the conserved components of ethylene signaling as those in Arabidopsis, including ethylene receptors, OsCTR2, OsEIN2, and OsEIL1. Besides the conserved receptor-CTR2-OsEIN2-OsEIL1 signaling pathway, our results suggest a novel MHZ1-AHP1/2-OsRR21 phosphorelay pathway, through which ethylene receptors could regulate the root growth of rice by suppressing the kinase activity of MHZ1. In the absence of ethylene, ethylene receptors are in active conformations, which may facilitate their interaction with MHZ1 and suppress MHZ1 activity. With ethylene, ethylene receptors possibly released the repression effect on MHZ1 and the phosphorelay pathway is activated. The MHZ1-mediated phosphorelay pathway and the OsEIN2-mediated phosphorelay path may work together to regulate a subset of downstream gene to modulate root growth. MHZ1 and OsRRs can also be transcriptionally induced by ethylene through the OsEIN2-regulated phosphorelay pathway, which may facilitate the maintenance of MHZ1-mediated signaling after activation. The orange arrow indicates transcriptional activation. In addition, H₂O₂ may also function through MHZ1 to participate in the ethylene-regulated root growth. Source data are provided as a Source Data file.

**Fig. 5 Genetic interaction of MHZ1 and OsEIN2-mediated pathways.** a Ethylene response of mhz1/OsEIN2-OE in comparison with mhz1 and OsEIN2-OE. Bars indicate 10 mm. b Quantification of root length of the mutants in a. Root lengths are means ± SD, n > 30. **P < 0.01; Student’s t-test. c Enlargement of root features of the mutants treated with 10 ppm ethylene in a. d Ethylene response of OsEIN2/MHZ1-OE in comparison with OsEIN2 and MHZ1-OE. Rice seedlings were grown in dark for 2 days with air or 10 ppm ethylene. Bars indicate 10 mm. e Quantification of root length (above) and relative root length (below) of the mutants in d. Data are means ± SD, n > 30. **P < 0.01; Student’s t-test. f Comparison of MHZ1-, OsEIN2-, and OsEIL1-regulated ethylene-response genes. Etiolated seedlings of WT, mhz1, OsEIN2 and OsEIL1 were grown for 2 days before treated with air or 10 ppm ethylene for 3 h. Roots were subjected to RNA-seq analysis with three biological replicates. Ethylene-response genes (ERGs) were identified in WT according to the gene expression levels with at least relative twofold changes (q-value < 0.05) in ethylene treatment compared to those in the air. A total of 1789 ERGs were identified. g A proposed working model for MHZ1-regulated ethylene signaling in rice. Rice has the conserved components of ethylene signaling as those in Arabidopsis, including ethylene receptors, OsCTR2, OsEIN2, and OsEIL1. Besides the conserved receptor-CTR2-OsEIN2-OsEIL1 signaling pathway, our results suggest a novel MHZ1-AHP1/2-OsRR21 phosphorelay pathway, through which ethylene receptors could regulate the root growth of rice by suppressing the kinase activity of MHZ1. In the absence of ethylene, ethylene receptors are in active conformations, which may facilitate their interaction with MHZ1 and suppress MHZ1 activity. With ethylene, ethylene receptors possibly released the repression effect on MHZ1 and the phosphorelay pathway is activated. The MHZ1-mediated phosphorelay pathway and the OsEIN2-mediated phosphorelay path may work together to regulate a subset of downstream gene to modulate root growth. MHZ1 and OsRRs can also be transcriptionally induced by ethylene through the OsEIN2-regulated phosphorelay pathway, which may facilitate the maintenance of MHZ1-mediated signaling after activation. The orange arrow indicates transcriptional activation. In addition, H₂O₂ may also function through MHZ1 to participate in the ethylene-regulated root growth. Source data are provided as a Source Data file.
AHK5, a homolog of MHZ1, is involved in the above pathway needs to be further investigated. Ethylene transcriptionally induces the expression of ethylene receptor genes ER5, ER52, and ETR2 in Arabidopsis11 and OsEtr2 in rice (Supplementary Data 1). It is possible that ethylene-induced receptor gene expression may function as a desensitizing approach at later stage for ethylene receptor to re-lock MHZ1 after the initial biochemical triggering of the signaling and completeness of ethylene response.

Downstream of ethylene receptors, two branch pathways are proposed (Fig. 5g). Apparently, the conserved OsCtRs-OsEIN2-OsEIL1 pathway should play major roles in both roots and aerial parts, whereas the MHZ1-OsAHP1/2-OsRR21 pathway may specifically play roles in roots rice. Ethylene-induced OsEIN2 accumulation is not affected in mhZ1 or mhZ1/OsEIN2-OE plants, further supporting a separate role of the MHZ1 in root ethylene response (Supplementary Fig. 13). Transcriptionally, MHZ1 and OsRR21 can be induced by the conserved OsEIN2-OsEIL1 pathway and this feature may facilitate maintenance of MHZ1-mediated signaling after activation (Fig. 2 and Supplementary Fig. 7g). In our RNA seq analysis, 719 ERGs were identified to be shared by MHZ1, OsEIN2 and OsEIL1 (Fig. 5f and Supplementary Data 1). It is proposed that the two pathways may work together to modulate the expression of these ERGs, thus regulating root growth. Interruption of either pathway would abolish the ethylene induction of these ERGs, causing an ethylene-insensitive phenotype of rice roots. On the other hand, when either pathway is interrupted, constitutive activation of the other pathway could partially restore the induction of downstream ERGs.

Two-component systems also participate in cytokinin signaling in Arabidopsis and similar mechanism may exist in rice38,64. Considering that rice may have only two functional HPTs (OsAHP1 and OsAHP2) for signaling47,68, it is hence possible that these two genes play roles in both ethylene signaling and cytokinin signaling in specific manners depending on distinct treatments, times, cell types, tissues and/or organs. Actually, only limited number of homozygous Osahp1 Osahp2 seedlings were identified from 168 self-bred progenies of the Osahp1 (heterozygous)/Osahp2 (homozygous) plant, implying that the Osahp1 Osahp2 double-mutant may have some degree of defect in embryo development, which may be caused by a defect in cytokinin signaling. Other possibilities cannot be excluded. Similar sharing of the histidine-containing phosphotransmitter has been reported in filamentous fungi Aspergillus nidulans64.

Arabidopsis has a MHZ1 homolog AHK5. Mutation of the gene caused mildly enhanced ethylene response in Arabidopsis ahk5 roots43. This response is in contrast with the complete ethylene-insensitive response in the present rice mhZ1 root, suggesting that rice may have evolved to adopt the MHZ1 pathway in a different mechanism to strongly control ethylene-regulated root growth for adaptation in water environment. ahk5 is also sensitive to ABA, and our present mhZ1 is slightly insensitive to ABA (Supplementary Fig. 14), suggesting that MHZ1 may also participate in ABA- or other stress-related processes in rice. A maize homolog ZmHK9 has been characterized and the gene is highly expressed in roots and induced by drought and ABA treatment44. Overexpression of the gene in transgenic Arabidopsis plants caused hypersensitivity to ABA and ethylene, and led to drought tolerance through regulation of stomatal density and stomatal closure44. These studies suggest that MHZ1 may also have other functions in addition to its roles in ethylene response.

In the N-terminal end of MHZ1, a PAS domain is noted (Supplementary Fig. 2c). This domain is usually involved in ligand binding and/or protein interaction, suggesting other sensing possibilities in addition to be regulated by ethylene receptors. The possibility that MHZ1 serves as a cytoplasmic co-receptor of ethylene signaling cannot be excluded since both MHZ1 and ethylene receptors are histidine kinases or structurally similar proteins. It should be noted that, Arabidopsis AHK5, several AHPs and response regulators can form signaling network to modulate stomatal closure in response to H2O2 and/or ethylene65-67. Root growth of mhZ1 is slightly insensitive to H2O2 (Supplementary Fig. 15), suggesting that H2O2 may partially function through MHZ1 to inhibit root growth of rice. Given that ethylene induces H2O2 production in Arabidopsis68, it is possible that H2O2 may also be involved in the proposed pathway (Fig. 5g). This result is consistent with the GO analysis that MHZ1-dependent ERGs are enriched in auxin signaling pathway and also responses to different stimuli. OsHK1/MHZ1 is previously reported to play roles in rice large radius root tip circumnutations through a cytokinin-related pathway52. As ethylene is also demonstrated to stimulate mutations in Arabidopsis in an auxin transport-dependent manner69, it is possible that MHZ1 is also involved in the crosstalk between ethylene and auxin, cytokinin or H2O2 to regulate root growth.

Collectively, we identified the histidine kinase MHZ1 as a regulator of ethylene signaling in rice. Ethylene receptor OsE RS2 interacts with MHZ1 through GAF domain and inhibits MHZ1-mediated signaling. The MHZ1-mediated pathway may function as a branch in parallel with the conserved pathway to regulate root growth especially under semi-aquatic environment (Fig. 5g). Our data provide valuable insights into the mechanism of ethylene signaling in rice and should facilitate improvement of stress adaptation and relevant agronomic traits in crops.

Methods

Materials, ethylene treatment, and gene identification. The rice (Oryza sativa L.) mutants mhZ1, Osein2/mhZ1-7, Osein2/mhZ6, and Oser2/mhZ12 mutants were previously identified by Ma31. OsEIN2-OE lines were generated by Ma41. All the mhZ1 alleles are in Nip background, and the Oser2 and Oser2 mutants are in D) background. Material propagation and crosses were carried out in the Experimental Station of the Institute of Genetics and Developmental Biology in Beijing from May to October of each year. For ethylene-response assay, seeds were soaked at 37°C for 2 days and the germinated seeds were placed on stainless net for ethylene treatment at 28°C in dark for 3 days if not specified, with a water level below the seeds31. Lengths of roots and/or coleoptiles were measured. ABA treatment, stock solution of ABA was prepared in ethanol and diluted into solutions of different concentrations with water. Equivalent volumes of ethanol were added to the control. The MHZ1 gene was identified by TAIL-PCR method. To generate MHZ1-OE lines, MHZ1 CDS driven by the native promoter (3 kb sequence upstream of ATG) was transformed into WT rice and homozygous transgenic lines with higher MHZ1 expression levels were analyzed. The ethylene receptor loss-of-function mutants Oser2 and Oser2 were purchased and identified through PCR with primers suggested (http://chik.hku.ac.kr/RISD_DB.html) (Supplementary Table 1). The mhZ1-1 was used as male parent and crossed with Oser2, Oser2, Oser2, and OsEIN2-OE transgenic lines to generate double mutants for genetic interaction analysis. The Oser2/MHZIOE 4-4 line was derived from crossing Oser2 with MHZIOE 4-4 and the Oser2/MHZIOE 3-5 line was generated by overexpressing MHZ1 in Oser2 mutant through transgenic approach. To generate the MHZ1-OE/Oser2 double-mutant, MHZ1-OE transgenic line was used as male parent and crossed with Oser2. F2 populations were used for genotyping and F3 or F4 populations were phenotyped. The expression of the mhZ6-15 mutant with the Tos17 insertion was requested from the rice mutant database in Japan (https://pc7080.abr.saff.cc/jp/-miyao/pub/tos17/index.html.en). The Osahp1, Osahp2 and Oser231 single mutants were generated through a CRISPR/Cas9 approach. SG sequences are as follows: Osahp1: GGGACAGATATCGTTATGAA. To generate Osahp1 double-mutant, Nip rice was transformed with vectors carrying two SG sequences targeting OsAHP1 and OsAHP2. After sequencing the genomic sequences of the two genes in 24 transgenic T0 lines, no Osahp1 Osahp2 double-mutant was identified. An Osahp1 plant was self-crossed and the self-bred progenies (168 seeds) were germinated and grown under 10 ppm ethylene. After phenotype observation, seedlings were numbered and OsAHPI and OsAHPI2 genomic sequences were sequenced. Homozygous Osahp1 Osahp2 plants were identified.
Gene expression analysis by real-time PCR. Two or three-day-old etiolated rice seedlings were treated with atr or ethylene before roots and shoots were harvested for RNA extraction. Total RNA was extracted using TRIZOL reagent (Invitrogen). The complementary DNAs (cDNAs) were synthesized using cDNA Synthesis Kit (M-MLV Version) (TaKaRa) and then subjected to real-time PCR. Real-time PCR was conducted according to the instructions of TransStart Green qPCR SuperMix (TransGen Biotech, China). OsActin2 was used for internal control. The primers are listed in Supplementary Table 1. The experiments were repeated independently for at least three times and the results were consistent. One set of results was used for analysis.

GUS Staining. Tissues and organs were fixed in 90% acetone on ice for 15 min. After washing 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), the samples were soaked in staining solution (staining containing 0.5 mg/mL X-Gluc (Sigma, B8040) for 10 min in a vacuum system. The samples were incubated at 37°C in the dark. Green tissues were decolorized with 70% ethanol. The samples were observed using stereo microscopy (Leica, M165 FC).

Proteins expression and phosphorylation assay. The cDNA fragments encoding various protein versions were fused with GST in pGEX-6P-1 vector or maltose-binding protein (MBP) gene in pMAL-2C vector and proteins were expressed in BL21 (DE3) pLysS. GST-MH21 (365–968 aa) containing kinase domain and receiver domain, and its mutant versions, GST-KD containing only the kinase domain (amino acids 1–123) were successfully expressed. Full-length MH21 (1–968 aa) was not successfully expressed. Four OsHPTs were expressed except OsHPT1 and OsHPT6 (543–968 aa) which were not successfully expressed. Four OsHPTs were expressed except OsHPT3 and OsHPT6 which were not successfully expressed.

For testing of the transactivation of the MH21 promoter activity in tobacco leaves, the open reading frame of OsEIL1 was cloned into pCambia2300-335-OC5 (generously provided by Dr. Kenji Ishii, University of California, USA, and Dr. Katsuhiko Ishii). The recombinant vector was used for Agrobacterium-mediated transformation of N. tabacum cv. Xanthi-NC89. The positive clones were selected on Kanamycin-containing media, followed by selection on X-gal agar plates. The transgenic plants were grown in earthen pots in climate chambers. In order to test the expression activity of MH21 promoter in rice organs, the coding sequences of MH21 were cloned into the rice promoter vector pBT3-STE (OsERS2-Cub) and MH21 into the rice vector pPR3-N (NubG-MH21) from the dual molecular marker kit (Dolivillo Biotech). The bait and prey constructs were then cotransformed into the yeast strain BY4741. Positive transformants were selected on 5-DFU-Leu medium, and protein–protein interactions were detected by Coomassie blue staining.

Pull-down of MBP-MH21 with GST-ERS2TM. To carry out the GST pull-down assay, the coding sequences of MH21 and OsERS2TM were cloned into the pMAL-c2 and pGEX-6P-1 plasmids, respectively, to make MBP-MH21 and GST-ERS2TM constructs. The constructs were then transformed into BL21(DE3). The transformed E. coli cells were cultured at 25°C and proteins were induced by addition of 0.2 mM IPTG. After sonication and centrifugation, lysate supernatants containing GST-ERS2TM recombinant protein were incubated with Glutathione sepharose at 4°C for 2 h. The supernatants were washed with phosphate-buffered saline (PBS, pH 7.3) for three times. The beads were incubated with 0.5 μM MBP-MH21 recombinant protein at 4°C for 2 h. The supernatants were washed with PBS (pH 7.3) for three times. Harvested beads were boiled with 2× SDS loading buffer before running the SDS-PAGE and immunoblotted with anti-GST, anti-MBP antibodies.

Co-IP assays. For coimmunoprecipitation of MH21 with ERS2TM, constructs containing MH21-FLAG and ERS2TM-GFP were cotransformed into rice protoplasts. Total proteins were extracted by homogenizing the protoplasts in 0.5 mL IP buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 50 μM MG132, 2% (v/v) protease inhibitor cocktail) and incubating the samples on ice for 15 min. The samples were centrifuged at 12,000 rpm for 10 min at 4°C before the supernatants were incubated with 25 μL of GFP-Trap beads for 2 h at 4°C. After being washed for three times with wash buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA), the beads were collected and resuspended with 50 μL of 2× SDS-PAGE loading buffer and heated at 95°C for 5 min. The eluted immunoprecipitates were immunoblotted with anti-GFP, anti-FLAG, and anti-UbGase antibodies. For interactions of MH21 with OsERS1 and OsETR2, constructs containing MH21-FLAG and ERS1TM-OERST/ETR2TM-GFP were cotransformed into rice protoplasts. Total proteins were immunoprecipitated with GFP-Trap and immunoblotted with anti-GFP, anti-FLAG, and anti-Actin antibodies.

Membrane recruitment assay. To perform the membrane recruitment assay, MH21-GFP and OsERS2-Flag-mCherry proteins were expressed separately or together in tobacco leaf epidermal cells and fluorescence was examined. The images were taken using a confocal microscopy (Zeiss LSM 710). Excitation/emission wavelengths were set at 488 nm/500–530 nm for GFP and 561 nm/582–639 nm for mCherry.

Histidine phosphorylation state detection. To detect the histidine phosphorylation state of MH21 in O. sativa 2 mutant, vector carrying MH21-FLAG was transformed into protoplasts of WT and Osers22 mutant. Total proteins were extracted and MH21-FLAG protein was immunoprecipitated with anti-FLAG affinity gel and immunoblotted with anti-FLAG or anti-IgG antibodies (Millipore, MAB1330).

Measurement of ethylene production. To test the ethylene production of different mutant backgrounds, seedlings were grown in 40 mL uncapped vials for 7 days in dark at 28°C. The
vials were then sealed with rubber syringe caps for 17 h. One millilitre of headspace of each vial was measured by using gas chromatography (GC-2014; Shimadzu). 33

Screening for suppressors of OsEIN2 (SOE). For generation of SOE lines, seeds of OsEIN2 overexpression line OsEIN2OE–44 were soaked in water for 16 h at room temperature. The seeds were then treated with 0.6% EMS (Sigma, M0880) for 8 h at room temperature. The EMS-treated seeds were germinated at 37 °C and grown in the field. M2 generation seeds of the EMS-mutagenized lines were used for mutant screening. 33

Statistical analysis. The relative root or coleoptile length of each mutant is analyzed relative to the length in untreated conditions. All of the data were analyzed using a one-way ANOVA (LSD test) for the test groups.

RNA-seq analysis. To carry out the RNA-seq analysis, etiolated seedlings of WT, mhz1, OsEIN2mhz7 and OsEII1mhz6 were grown in the dark at 28 °C for 2 days before treated with air or 10 ppm ethylene for 3 h. Roots of WT and different mutants were subjected to RNA-seq analysis with three biological replicates. The clean data was mapped to rice genome by TopHat and analyzed with Cufflinks software. In WT and different mutants, genes with at least twofold changes in expression relative to the length in untreated conditions. All of the data were analyzed using a one-way ANOVA (LSD test) for the test groups.

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