Membrane protein MHZ3 stabilizes OsEIN2 in rice by interacting with its Nramp-like domain

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The phytohormone ethylene regulates many aspects of plant growth and development. EIN2 is the central regulator of ethylene signaling, and its turnover is crucial for triggering ethylene responses. Here, we identified a stabilizer of OsEIN2 through analysis of the rice ethylene-response mutant *mhz3*. Loss-of-function mutations lead to ethylene insensitivity in etiolated rice seedlings. *MHZ3* encodes a previously uncharacterized membrane protein localized to the endoplasmic reticulum. Ethylene induces *MHZ3* gene and protein expression. Genetically, *MHZ3* acts at the *OsEIN2* level in the signaling pathway. MHZ3 physically interacts with OsEIN2, and both the N- and C-termini of MHZ3 specifically associate with the OsEIN2 Nramp-like domain. Loss of *mhz3* function reduces OsEIN2 abundance and attenuates ethylene-induced OsEIN2 accumulation, whereas *MHZ3* overexpression elevates the abundance of both wild-type and mutated OsEIN2 proteins, suggesting that MHZ3 is required for proper accumulation of OsEIN2 protein. The association of MHZ3 with the Nramp-like domain is crucial for OsEIN2 accumulation, demonstrating the significance of the OsEIN2 transmembrane domains in ethylene signaling. Moreover, MHZ3 negatively modulates OsEIN2 ubiquitination, protecting OsEIN2 from proteasome-mediated degradation. Together, these results suggest that ethylene-induced MHZ3 stabilizes OsEIN2 likely by binding to its Nramp-like domain and impeding protein ubiquitination to facilitate ethylene signal transduction. Our findings provide insight into the mechanisms of ethylene signaling.

ethylene | protein stabilization | protein–protein interaction | rice

The phytohormone ethylene regulates multiple aspects of plant growth and development. A linear signaling pathway has been established based on extensive studies in *Arabidopsis* (1). Ethylene is perceived by a family of endoplasmic reticulum (ER) membrane-bound receptors (2–5). The signal is transmitted through the negative regulator CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) (6) and then the positive regulator ETHYLENE INSENSITIVE 2 (EIN2) (7) and is further amplified via the master transcription factor EIN3 and EIN3-LIKE1 (EIL1)-mediated transcription cascades, which ultimately activate a great deal of ethylene-responsive genes (8–10).

EIN2 is the essential regulator of the ethylene response (7). The N terminus of EIN2 consists of 12 predicted transmembrane helices that show sequence similarity to the Nramp family of metal transporters, and its C terminus has a large hydrophilic domain (7). EIN2 is predominantly localized to the ER (11). In the absence of ethylene, the receptors negatively regulate ethylene responses by activating downstream CTR1 (12). The Raf-like Ser/Thr kinase CTR1 directly phosphorylates EIN2 to prevent it from signaling (13). F-box proteins EIN2-TARGETING PROTEIN1 (ETP1) and ETP2 interact with the EIN2 C terminus and target the protein for proteasome-mediated degradation (14). In the presence of ethylene, unphosphorylated EIN2 undergoes proteolytic cleavage, and the cytosolic C-terminal domain is translocated into the nucleus (13, 15, 16). In the nuclei, EIN2 directly regulates histone acetylation to facilitate EIN3 binding to its target genes (17, 18). Interestingly, the EIN2 C-terminal domain can also be transferred into the P-body to mediate translational repression of EIN3-BINDING F-BOX PROTEIN1 (EBF1) and EBF2 (19, 20), which target EIN3/EIL1 for degradation in the absence of ethylene (21, 22). Extensive studies have elucidated how EIN2 activates downstream signaling through its C-terminal domain; however, the significance of the N-terminal Nramp-like domain is largely unknown.

Rice (*Oryza sativa*) is a monocotyledonous model plant. In comparison with *Arabidopsis*, rice exhibits different features in many aspects such as plant structure, living environment, growth and developmental process, and ethylene responses (23). Importantly, rice has limited synteny with *Arabidopsis* at the genome level (24). These facts suggest that rice can be used as an alternative system for the identification of novel components of ethylene signaling. We have developed a mutant screening system in rice and isolated a set of ethylene-response mutants (25–30). In this study, we characterized the ethylene-insensitive *mhz3* mutant. *MHZ3* encodes a previously uncharacterized ER membrane protein that genetically acts on and physically interacts with OsEIN2. MHZ3 associates with the OsEIN2 Nramp-like domain to stabilize the protein by inhibiting ubiquitination and

Significance

The ethylene signaling pathway has been extensively investigated in *Arabidopsis*, and EIN2 is the central component. Rice is a monocotyledonous model plant that exhibits different features in many aspects compared with the dicotyledonous *Arabidopsis*. Thus, rice provides an alternative system for identification of novel components of ethylene signaling. In this study, we identified a stabilizer of OsEIN2 through analysis of the rice ethylene-insensitive mutant *mhz3*. We found that MHZ3 stabilizes OsEIN2 likely by binding to its Nramp-like transmembrane domain and impeding protein ubiquitination, blocking proteasome-mediated protein degradation. This study reveals that MHZ3 is required for ethylene signaling and identifies how MHZ3 binds to OsEIN2 via the OsEIN2 N-terminal Nramp-like domain.

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proteasome-mediated degradation. Our findings reveal a potential mechanism by which EIN2 transduces ethylene signals via its N-terminal Nramp-like domain.

Results

Phenotypic Analysis and Gene Identification of *mhz3* Mutant. Five allelic *mhz3* mutants (*mhz3-1* to *mhz3-5*) were previously identified in our genetic screen for rice ethylene-response mutants (25). Ethylene treatment inhibited root growth but promoted coleoptile elongation in dark-grown wild-type (WT) rice seedlings (Fig. 1*A*). By contrast, the effects of ethylene on coleoptile and root growth of etiolated rice seedlings are almost completely blocked by the *mhz3* mutation (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*). Treatment with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, reduced coleoptile length and increased root length in WT seedlings due to repression of the activity of endogenous ethylene. However, 1-MCP treatment has no effects on *mhz3* seedlings (Fig. 1*B*). These results suggest that *mhz3* is insensitive to both endogenous and exogenous ethylene. The ethylene responsiveness of *mhz3* was further confirmed at the molecular level by examining the expression of ethylene-inducible genes, and ethylene induction of the genes was abolished or hampered in the *mhz3* mutant (Fig. 1*C*).

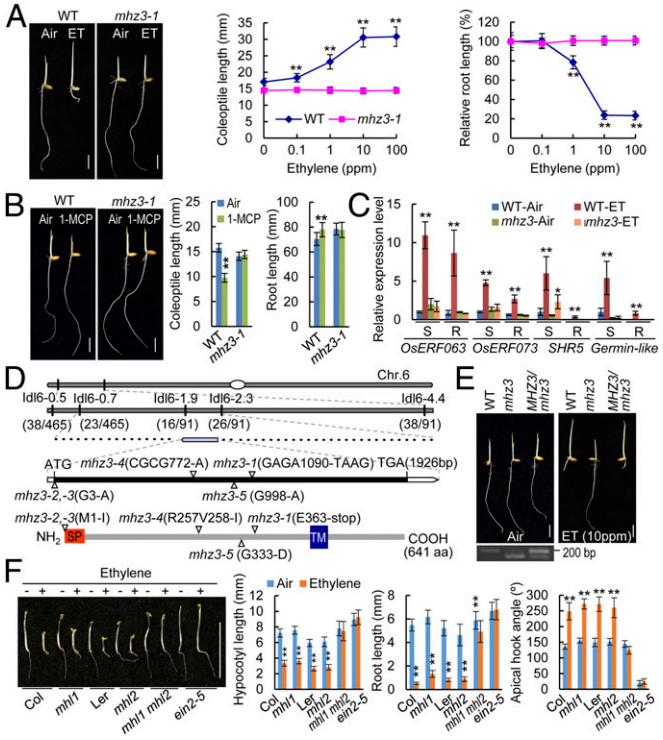

Fig. 1. Phenotypic analysis and gene identification of *mhz3*. (*A*) Ethylene-response phenotype of *mhz3*. Etiolated seedlings were treated with various concentrations of ethylene for 3 d. Representative seedlings grown in the air and in 10 ppm ethylene (ET) are shown (*Left*). Coleoptile (*Center*) and root lengths (*Right*) are means ± SD, $n > 30$ (**$P < 0.01$; Student's *t* test; compared with 0 ppm). (*B*) *mhz3* is insensitive to 1-MCP. (*Left*) Etiolated seedlings were treated with 10 ppm 1-MCP or without (Air) for 3 d. Coleoptile (*Center*) and root lengths (*Right*) are means ± SD ($n > 30$). Asterisks indicate significant difference between Air and 1-MCP ($P < 0.01$; Student's *t* test). (*C*) Ethylene-induced gene expression is abolished in *mhz3*. Total RNAs from etiolated 2-d-old seedlings treated with 10 ppm ethylene or without (Air) for 8 h were subjected to qRT-PCR analysis. Data are means ± SD, $n = 3$ (*$P < 0.05$, **$P < 0.01$; Student's *t* test; compared with Air). R, root; S, shoot. (*D*) Map-based cloning of *MHZ3*. The mutation sites are indicated in schematic diagrams. (*E*) Functional complementation of *mhz3-1* with WT *MHZ3* genomic DNA (*Upper*). (*Lower Left*) Confirmation of the transgene by PCR. (*F*) Triple response of *mhl1 mhl2* double mutant of *Arabidopsis*. (*Left*) Etiolated seedlings were treated with (+) or without (–) 10 ppm ethylene for 4 d. (*Right*) Data are means ± SD, $n > 15$ ($P < 0.01$; Student's *t* test; compared with "Air"). (*A*, *B*, *E*, and *F* scale bars, 10 mm.)

The *MHZ3* gene was identified by map-based cloning. All *mhz3* mutants harbored a mutation at the LOC_Os06g02480 locus (Fig. 1*D*). The mutation sites were further confirmed by PCR-based analysis using cleaved amplified polymorphic sequence (CAPS) or derived CAPS primers (*SI Appendix*, Fig. S1*C*). Genetic transformation with the WT genomic DNA fragment rescued the ethylene-insensitive phenotype of *mhz3-1* (Fig. 1*E*). The results convincingly demonstrate that *MHZ3* is located at the LOC_Os06g02480 locus. Gene expression and promoter-GUS analyses revealed that *MHZ3* is universally expressed in the rice organs examined from vegetative to reproductive stages (*SI Appendix*, Fig. S2). The deduced MHZ3 protein has no domains of known function, except for a signal peptide (SP) and one transmembrane helix (TM) (Fig. 1*D*). BLAST search and phylogenetic analysis revealed that MHZ3 belongs to a previously uncharacterized plant-specific gene family that is distributed from algae to land plants (*SI Appendix*, Figs. S3 and S4). Ortholog search in the Rice Genome Annotation Project database (rice.plantbiology.msu.edu/) reveals that *MHZ3* is a single-copy gene in the rice genome but has two copies in the *Arabidopsis* genome. We named these *Arabidopsis* homologous genes *MHZ3-Like1* (*MHL1*, At1g75140) and *MHL2* (At1g19370), which share 40% and 38% identity with MHZ3, respectively. Although the *mhl1* and *mhl2* single mutants exhibited WT-like ethylene response likely due to functional redundancy, the *mhl1 mhl2* double mutant displayed ethylene-insensitive phenotype, indicating that *MHL1* and *MHL2* are required for ethylene signaling in *Arabidopsis* (Fig. 1*F* and *SI Appendix*, Fig. S5 *A*–*C*). However, it appears that the ethylene-insensitive phenotype of the double mutant is weak compared with that of *ein2-5* mutant, suggesting that the function of *MHZ3*-like genes may not be as strong as that of *EIN2* in ethylene signaling.

MHZ3 Is an ER-Localized Glycosylated Membrane Protein. We generated a specific anti-MHZ3 antibody for detecting the endogenous protein. Total proteins of WT seedlings were separated into soluble and microsomal fractions by ultracentrifugation. MHZ3 was mainly detected in the microsomal fraction, indicating that MHZ3 is a membrane-bound protein (Fig. 2*A*). The microsomal membranes were further separated into plasma membrane (upper phase) and endomembrane systems (lower phase) by aqueous two-phase partitioning. MHZ3 was solely detected in the lower phase, indicating an endomembrane localization of MHZ3 (Fig. 2*B*). To further determine the subcellular localization, we transiently expressed MHZ3-GFP in tobacco leaf cells. GFP fluorescence was mainly detected in a reticular network-like structure that is labeled by the ER marker protein mCherry-HDEL, suggesting that MHZ3 is predominantly localized at the ER (Fig. 2*C*). In addition, we observed fluorescent signals in distinct dots along with the ER networks (Fig. 2*C*), implying that MHZ3 may also target to organelles/compartments other than the ER. Its localization outside the ER may suggest that MHZ3 has additional functions in ethylene signaling and/or in other processes. The MHZ3 protein is predicted to contain three potential *N*-glycosylation sites, and two of these are conserved in all species (*SI Appendix*, Fig. S3). Deglycosylation assay detected *N*-glycosylation modification of MHZ3 (Fig. 2*D*).

MHZ3 Expression Is Induced by Ethylene, and the Gene Overexpression Confers Ethylene Hypersensitivity. We examined *MHZ3* gene expression in response to ethylene. The transcript level of *MHZ3* was significantly induced by ethylene treatment in both shoots and roots of etiolated WT seedlings, whereas the induction was largely blocked in *Osein2-1*, *Osers2*^{d} (a dominant ethylene receptor gain-of-function mutation), and *Oseil1* seedlings (Fig. 2*E*). In *Arabidopsis*, the transcript level of *MHL2* was also significantly induced by ethylene, whereas *MHL1* expression was unaffected by ethylene

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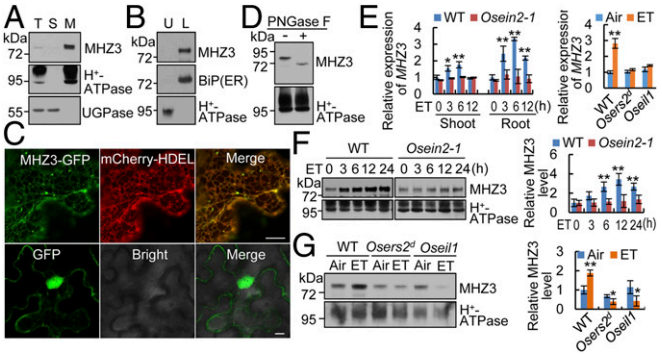

Fig. 2. MHZ3 is an ER-localized glycosylated membrane protein, and its expression is induced by ethylene. (*A*) Membrane association of MHZ3. Equal amounts of total protein (T), soluble protein (S), and microsomal membranes (M) were immunoblotted for MHZ3, H^+-ATPase (plasma membrane marker), and UGPase (cytoplasm marker). (*B*) Endomembrane association of MHZ3. Microsomal membranes were separated into plasma membrane (upper phase; U) and endomembrane (lower phase; L) systems and then immunoblotted for MHZ3, BiP, and H^+-ATPase. (*C*) ER localization of MHZ3 as revealed by transient expression of MHZ3-GFP in tobacco leaf epidermal cells. mCherry-HDEL is used as an ER marker. GFP is used as a control. (Scale bars, 10 μm.) (*D*) Deglycosylation assay of MHZ3 using PNGase F. (*E*) *MHZ3* transcript level is induced by 10 ppm ethylene (ET) as revealed by qRT-PCR analysis. Data are means ± SD, $n = 3$ [$^*P < 0.05$, $^{**}P < 0.01$; Student's *t* test; compared with 0 h (*Left*) or Air (*Right*)]. (*F* and *G*) MHZ3 protein level is induced by ethylene, but the induction is impaired in ethylene-insensitive mutants. (*Left*) Membrane proteins isolated from rice shoots (*F*) or roots (*G*) of etiolated seedlings were immunoblotted for MHZ3 and H^+-ATPase (loading control). (*Right*) Statistical analysis of the relative MHZ3 levels from three independent replicates is presented, and the data are means ± SD, $n = 3$ [$^*P < 0.05$, $^{**}P < 0.01$; Student's *t* test; compared with 0 h (*F*) or Air (*G*)].

treatment (*SI Appendix*, Fig. S5*D*). The protein level of MHZ3 increased steadily upon ethylene treatment (Fig. 2*F*). However, the ethylene-induced accumulation of MHZ3 protein was completely abolished in *Osers2*^{d}, *Osein2-1*, and *Oseil1* mutants (Fig. 2 *F* and *G*). These results suggest that MHZ3 expression is induced by ethylene at both transcriptional and protein levels and that the induction requires an intact ethylene signaling pathway. Notably, ethylene treatment significantly reduced the MHZ3 level in *Osers2*^{d} and *Oseil1* mutants. This may be due to the depletion of basal MHZ3 protein in stabilizing OsEIN2 (as revealed in the following sections) in the presence of ethylene. Furthermore, we examined MHZ3 localization under ethylene treatment and in *Osein2* null backgrounds. Introduction of *35S:MHZ3-GFP* into *mhz3-1* plants rescued the mutant phenotype, indicating that GFP tagging does not affect MHZ3 function (*SI Appendix*, Fig. S6*A*). The localization pattern of MHZ3 as revealed by GFP fluorescence is not altered upon ethylene treatment (*SI Appendix*, Fig. S6*B*). The *35S:MHZ3-GFP* and *35S:mCherry-HDEL* constructs were cotransformed into rice protoplasts of *Osein2* null mutants, and the *OsEIN2* mutations did not affect MHZ3 localization (*SI Appendix*, Fig. S6*C*).

Next, we overexpressed *MHZ3* in WT rice plants to study gene function (*SI Appendix*, Fig. S7 *A* and *B*). Compared with the WT and low-expression line (OX26), the high-expression lines (OX21, OX22, and OX24) exhibited slightly but significantly longer coleoptiles and shorter roots when grown in the air and displayed strong inhibition of root growth upon ethylene treatment (*SI Appendix*, Fig. S7*C*). 1-MCP treatment only partially reduced the constitutive ethylene response of *MHZ3*-OX lines (*SI Appendix*, Fig. S7*C*). The results suggest that *MHZ3* overexpression confers ethylene hypersensitivity in etiolated rice seedlings. At adult stages, *MHZ3* overexpression altered some important agricultural traits including reduction of plant height, promotion of leaf senescence, and enhancement of grain size and grain weight (*SI Appendix*, Fig. S8).

***MHZ3* Genetically Acts at *OsEIN2*.** Genetic analyses were performed to position MHZ3 in the ethylene signaling pathway. Double-mutant analysis showed that ethylene hypersensitivity in the roots of *Osetr2* and *Osers2* ethylene-receptor loss-of-function mutants was completely abolished by *mhz3* mutation, indicating that *MHZ3* may act at or downstream of ethylene receptors (Fig. 3*A*). To examine the epistatic relationship between *MHZ3* and *CTR1*, we constructed the *mhl1 mhl2 ctr1* triple mutant of *Arabidopsis*. The constitutive signaling caused by *ctr1* mutation was fully suppressed by *mhl1 mhl2* double knockout, implying that *MHZ3* may act at or downstream of *ctr1* (*SI Appendix*, Fig. S9). Next, we examined the genetic interaction between *MHZ3* and *OsEIN2*. Overexpression of *OsEIN2* in WT seedlings resulted in strong constitutive and enhanced ethylene responses (Fig. 3*B*) (25). In contrast, *OsEIN2* overexpression in *mhz3* background led to a weak constitutive ethylene response in the air, but no further response to ethylene treatment (Fig. 3*B*). The results reveal that the effect of *OsEIN2* on rice ethylene response depends on *MHZ3* function, suggesting that *MHZ3* may act at or downstream of *OsEIN2*. We previously identified two types of *mhz7*/*Osein2* allelic mutants (25). *Osein2-1* harbors an 8-aa-deletion in the loop 2 located between the second and third transmembrane helices in the Nramp-like domain, and *Osein2-2* contains a premature stop codon in the nuclear localization signal located at the C-terminal end (25). Interestingly, overexpression of *MHZ3* in *Osein2-1* fully suppressed its ethylene insensitivity (Fig. 3*C*). However, *MHZ3* overexpression in *Osein2-2* was unable to suppress the mutant phenotype (Fig. 3*D*). Together, these results suggest that *MHZ3* likely acts at the *OsEIN2* level in the signaling pathway. Next, we further examined the epistatic relationship between *MHZ3* and *OsEIL1*, which mainly regulates rice root ethylene responses as previously identified (26). Overexpression of *MHZ3* in *Oseil1* was unable to rescue the mutant phenotype (Fig. 3*E*). In contrast, overexpression of *OsEIL1* in *mhz3* led to strong inhibition of root growth in the absence of ethylene, which was indistinguishable from that conferred by *OsEIL1* overexpression in WT (Fig. 3*F*). These results indicate that *MHZ3* likely functions upstream of *OsEIL1*. On the other hand, in the presence of ethylene, the *35S:OsEIL1*/*mhz3* seedlings did not show any further ethylene response relative to the seedlings of *35S:OsEIL1*/WT, which exhibited shorter roots and longer coleoptiles upon ethylene treatment (Fig. 3*F*), indicating that *MHZ3* is required for ethylene-induced OsEIL1 activity. Considering the fact that ethylene-induced EIN3/EIL1 stabilization fully relies on the presence of the upstream EIN2 protein (31), our observations agree that *MHZ3* works at the *OsEIN2* level to modulate OsEIL1 activity.

To further validate the genetic relationship between MHZ3 and OsEIN2, we compared their downstream ethylene-response genes (ERGs) identified by RNA-seq analysis (National Center for Biotechnology Information Sequence Read Archive, accession no. SRP041468). In rice shoots, 97.2% (3,702) of *MHZ3*-dependent ERGs were regulated by *OsEIN2*; in the roots, 81.5% (682) of *MHZ3*-dependent ERGs were regulated by *OsEIN2* (*SI Appendix*, Fig. S10). The results indicate that *MHZ3* and *OsEIN2* regulate a similar subset of downstream ERGs. Collectively, genetic analyses demonstrate that *MHZ3* likely acts at the *OsEIN2* level in the signaling pathway.

MHZ3 Physically Interacts with OsEIN2 via Association with Its N-Terminal Nramp-Like Domain. InterPro Domain Scan (www.ebi.ac.uk/interpro/) analysis revealed that the N terminus of MHZ3 has a domain (amino acids 139 to 458) belonging to a seven-bladed WD β-propeller superfamily that facilitates protein binding, implying that MHZ3 may function through protein–protein interaction. To test this possibility, we performed a membrane-based yeast two-hybrid (Y2H) assay to examine the interaction of MHZ3 with OsEIN2. In comparison with the negative controls coexpressing the bait or prey with empty vectors, the yeast cells

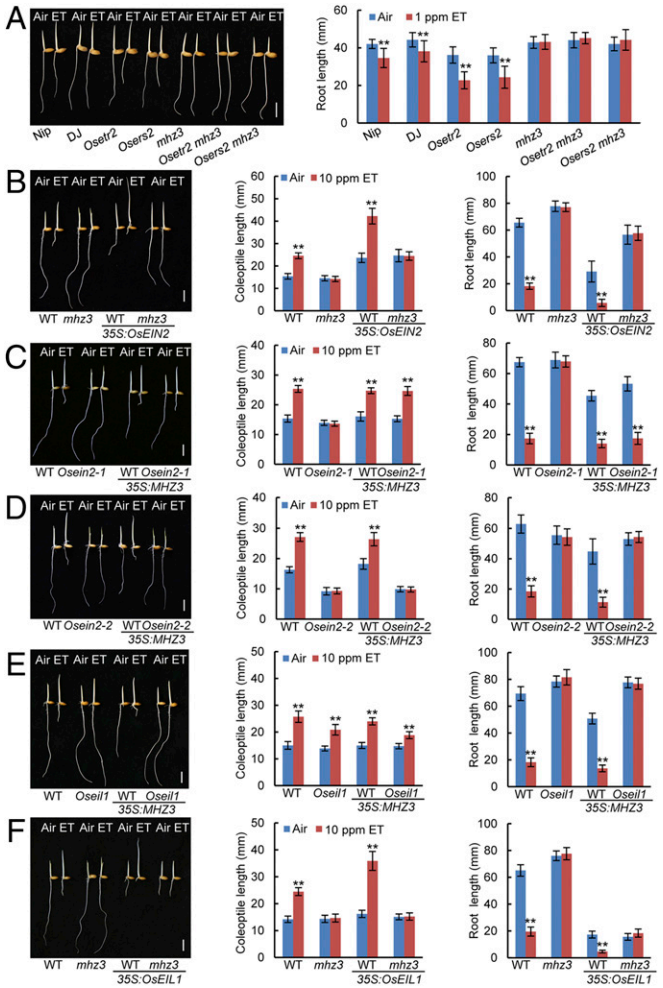

Fig. 3. MHZ3 genetically acts at OsEIN2 in ethylene signaling pathway. (*A*) Ethylene hypersensitivity caused by *Osetr2* and *Osers2* loss-of-function mutations is fully suppressed by *mhz3*. (*Left*) Etiolated seedlings were grown in the air or 1 ppm ethylene (ET) for 2 d. (*Right*) Root lengths were means ± SD, $n = 30$ (**$P < 0.01$; Student's *t* test; compared with Air). DJ, Dongjin; Nip, Nipponbare. (*B*) *OsEIN2* overexpression could not restore the ethylene response of *mhz3*. (*Left*) Etiolated seedlings were grown in the air or 10 ppm ethylene for 3 d. Coleoptile (*Center*) and root lengths (*Right*) are means ± SD, $n > 30$ (**$P < 0.01$; Student's *t* test; compared with Air). The ethylene response assays were the same in *B*–*F*. (*C*) Overexpression of *MHZ3* in *Osein2-1* rescued the mutant phenotype. (*D*) Overexpression of *MHZ3* in *Osein2-2* could not rescue the mutant phenotype. (*E*) Overexpression of *MHZ3* in *Oseil1* could not rescue the mutant phenotype. (*F*) Overexpression of *OsEIL1* in *mhz3* results in constitutive ethylene response, but no further response to ethylene treatment. (Scale bars, 10 mm.)

coexpressing MHZ3-Cub and NubG-OsEIN2 were able to grow on the selective media (Fig. 4*A*), suggesting that MHZ3 can directly interact with OsEIN2 in yeast cells. To validate the protein–protein interaction, we performed a coimmunoprecipitation (Co-IP) assay in transgenic rice plants stably expressing *OsEIN2-GFP*. MHZ3 protein was strongly coimmunoprecipitated by OsEIN2-GFP, but not by the GFP tag, suggesting that MHZ3 associates with OsEIN2 *in planta* (Fig. 4*B*). The MHZ3–OsEIN2 interaction was further confirmed using bimolecular fluorescence complementation (BiFC) assays in tobacco leaf epidermal cells and in rice protoplasts (*SI Appendix*, Fig. S11).

Next, we performed interaction domain mapping by using Co-IP assay in rice protoplasts of *Osein2 mhz3* double-knockout mutant. MHZ3 was divided into N- terminal (MHZ3-N) and C-terminal (MHZ3-C) halves, both of which contained the SP and TM to ensure proper membrane topology of the truncated proteins (Fig. 4*C*). OsEIN2 was divided into Nramp-like domain (OsEIN2-N) and C-terminal domain (OsEIN2-C) (Fig. 4*C*). MHZ3-N and MHZ3-C strongly coprecipitated with OsEIN2 (Fig. 4*D*, *Left*). Unexpectedly, both MHZ3-N and MHZ3-C associated with OsEIN2-N, but not with OsEIN2-C (Fig. 4*D*, *Center* and *Right*). Interaction domain mapping was confirmed by BiFC assays in rice protoplasts (*SI Appendix*, Fig. S11*B*). The results suggest that MHZ3 interacts with OsEIN2 by associating with its Nramp-like domain. Interestingly, MHZ3-N can also interact with the mutated Osein2-1-N (Fig. 4*D*, *Center* and *SI Appendix*, Fig. S11*B*). This provides a potential molecular basis for recovery of the ethylene response of *Osein2-1* by *MHZ3* overexpression.

MHZ3 Is Required for Proper Accumulation of OsEIN2. Given that MHZ3 physically interacts with OsEIN2, we examined the effects of MHZ3 on OsEIN2 activity. GFP tagging does not affect OsEIN2 function (*SI Appendix*, Fig. S12*A*). A similar ER localization pattern of OsEIN2 was observed in the WT and *mhz3* protoplasts transiently expressing *OsEIN2-GFP*, suggesting that MHZ3 does not influence the subcellular localization of OsEIN2, although the percentage of fluorescent cells in the *mhz3* background is obviously lower as compared with WT (*SI Appendix*, Fig. S12*B*). Moreover, nucleus-localized OsEIN2 C-terminal fragments were detected in both WT and *mhz3* plants stably expressing the *OsEIN2-GFP* transgene, indicating that MHZ3 does not affect the nuclear translocation of OsEIN2 C-terminal domain (*SI Appendix*, Fig. S12*C*). Gene expression analysis revealed that neither *mhz3* mutation nor *MHZ3* overexpression significantly altered the transcript levels of *OsEIN2* compared with those in WT seedlings (*SI Appendix*, Fig. S13), suggesting that MHZ3 does not affect *OsEIN2* expression at the transcriptional level.

Next, we examined the effects of MHZ3 on OsEIN2 protein level. We generated a specific anti-OsEIN2 antibody to detect the endogenous OsEIN2 protein. In WT seedlings, the level of OsEIN2 protein was apparently elevated by ethylene treatment

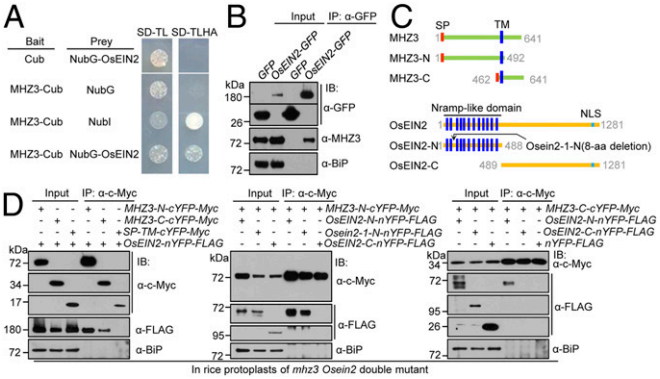

Fig. 4. MHZ3 physically interacts with OsEIN2 through association with its N-terminal Nramp-like domain. (*A*) Split-ubiquitin Y2H assay for interaction of MHZ3 and OsEIN2. NubI is the WT N-terminal half of ubiquitin and serves as positive control. (*B*) Co-IP of MHZ3 with OsEIN2 *in planta*. Transgenic rice seedlings stably expressing *35S:OsEIN2-GFP* or *35S:GFP* were treated with 10 ppm ethylene for 3 d. Total proteins were immunoprecipitated with GFP-Trap and immunoblotted with anti-GFP, anti-MHZ3, and anti-BiP (internal control) antibodies. (*C*) Diagrams of full-length and truncated versions of MHZ3 (*Upper*) and OsEIN2 (*Lower*) used in interaction domain mapping studies. (*D*) Co-IP assays for interaction domain mapping of MHZ3 and OsEIN2. The *SP-TM-cYFP-Myc* construct contains SP and TM of MHZ3 and was used as a negative control. The constructs were cotransformed into rice protoplasts. Total proteins were immunoprecipitated with anti-Myc affinity gel and immunoblotted with anti-c-Myc, anti-FLAG, and anti-BiP antibodies.

(Fig. 5*A*, *Left*). However, in *mhz3* seedlings, OsEIN2 was nearly undetectable at most time points, and only a slight accumulation of the protein was observed after 24 h of ethylene treatment (Fig. 5*A*, *Left*). Similarly, in the *OsEIN2*-overexpressing line in the WT background, the OsEIN2 level gradually increased upon ethylene treatment, but in the *mhz3* background, OsEIN2 was detected at very low levels, and no detectable accumulation of the protein was observed upon ethylene treatment (Fig. 5*A*, *Right*). The results suggest that *mhz3* mutation reduces the basal OsEIN2 level and attenuates ethylene-induced OsEIN2 accumulation. Furthermore, we tested the effect of *MHZ3* overexpression on OsEIN2 protein level in WT, *Osein2-1*, and *Osein2-2* backgrounds using the transgenic lines in Fig. 3 *C* and *D*. *MHZ3* overexpression markedly elevated OsEIN2 abundance in WT seedlings (Fig. 5*B*). Similarly, overexpression of *MHZ3* in *Osein2-1* and *Osein2-2* backgrounds apparently enhanced accumulation of the Osein2 mutant proteins which were almost undetectable in the single mutants (Fig. 5*B*). The results suggest that *MHZ3* overexpression facilitates the accumulation of both WT and mutated OsEIN2 proteins. Together, our data demonstrate that MHZ3 is indispensable for the proper accumulation of OsEIN2 protein.

The Association of MHZ3 with OsEIN2 Nramp-Like Domain Is Crucial for OsEIN2 Accumulation. To evaluate the specificity of the MHZ3-OsEIN2 interaction for OsEIN2 accumulation, we examined the effect of MHZ3 on the accumulation of the OsEIN2 C-terminal domain, which could not interact with MHZ3 as shown earlier (Fig. 4*D*). GFP-tagged full-length OsEIN2 and Osein2-1 and the truncated OsEIN2 C-terminal domain were transiently expressed in rice protoplasts isolated from *mhz3* mutant and *MHZ3*-OX (OX22) seedlings. The full-length OsEIN2 and Osein2-1 proteins obviously accumulated when expressed in OX22 protoplasts compared with those in the *mhz3* mutant (Fig. 5*C*). This is consistent with the observations in stable transgenic plants (Fig. 5*B*). By contrast, no significant difference in the protein levels of the truncated OsEIN2 C-terminal domain was detected when expressed in *mhz3* mutant and OX22 backgrounds (Fig. 5*C*). These results suggest that the N-terminal Nramp-like domain is required for the MHZ3-mediated accumulation of OsEIN2 protein. Consequently, it can be concluded that the binding of MHZ3 to the OsEIN2 Nramp-like domain is crucial for OsEIN2 accumulation.

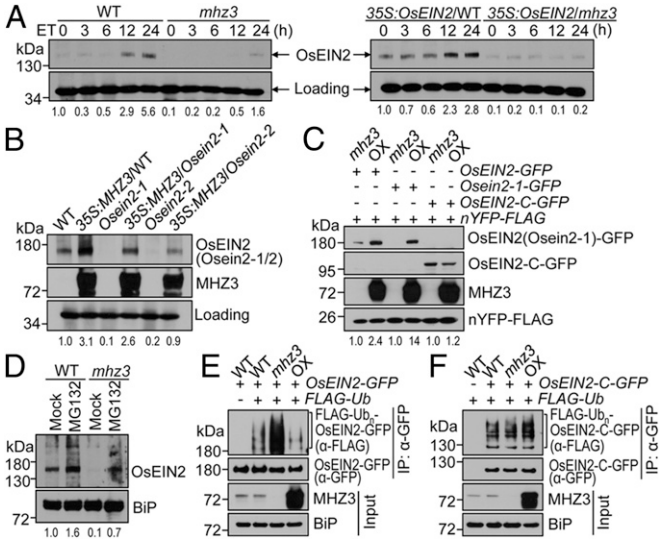

Fig. 5. MHZ3 is required for OsEIN2 accumulation and impedes the protein ubiquitination. (*A*) Ethylene-induced OsEIN2 accumulation is impaired by *mhz3* mutation. Etiolated 2-d-old seedlings of WT, *mhz3*, and *35S:OsEIN2* transgenic lines (the same lines as in Fig. 3*B*) were treated with 10 ppm ethylene for 0 to 24 h. Total proteins were immunoblotted for OsEIN2. A nonspecific band was used as a loading control. The values at the bottom indicate averages of relative OsEIN2 levels from three independent replicates; this is same in *B*–*D*. (*B*) *MHZ3* overexpression elevates OsEIN2 abundance. Total proteins isolated from etiolated 2-d-old seedlings of WT, *Osein2* mutants, and *35S:MHZ3* transgenic lines (the same lines as in Fig. 3 *C* and *D*) were immunoblotted for OsEIN2 and MHZ3. Others are as in *A*. (*C*) The Nramp-like domain of OsEIN2 is crucial for MHZ3-mediated OsEIN2 accumulation. The constructs were cotransformed into *mhz3* and *MHZ3*-OX22 (OX) protoplasts. *nYFP-FLAG* served as an internal control for normalizing the transformation efficiency. Total proteins were immunoblotted with anti-GFP, anti-FLAG, and anti-MHZ3 antibodies. (*D*) OsEIN2 protein in *mhz3* mutant is stabilized by MG132. (*E* and *F*) Ubiquitination analysis for OsEIN2 (*E*) and OsEIN2-C (*F*) in different MHZ3 backgrounds. The constructs were cotransformed into rice protoplasts and incubated in the presence of 3 μM MG132 for 16 h. Total proteins were immunoprecipitated using GFP-Trap and immunoblotted with anti-FLAG and anti-GFP antibodies. Input proteins were immunoblotted for MHZ3 and BiP (loading control).

MHZ3 Impedes OsEIN2 Ubiquitination. To explore the underlying mechanisms by which MHZ3 stabilizes OsEIN2, we treated *mhz3* seedlings with the proteasome inhibitor MG132. The treatment partially restored OsEIN2 accumulation in *mhz3* mutant, suggesting that MHZ3 modulates OsEIN2 accumulation through the proteasomal pathway (Fig. 5*D*). Next, we examined the ubiquitination of OsEIN2 in different MHZ3 backgrounds using the protoplast transient expression system. Compared with that in WT protoplasts, the ubiquitination level of OsEIN2 was obviously enhanced in *mhz3* mutant but suppressed in *MHZ3*-OX backgrounds, suggesting that MHZ3 negatively modulates OsEIN2 ubiquitination (Fig. 5*E*). In comparison with the full-length OsEIN2, no significant difference in the ubiquitination level of the OsEIN2 C-terminal domain was detected in WT, *mhz3*, and *MHZ3*-OX backgrounds (Fig. 5*F*), implying that the N-terminal Nramp-like domain of OsEIN2 is required for MHZ3-modulated OsEIN2 ubiquitination. Collectively, our results suggest that MHZ3 stabilizes OsEIN2 likely by binding to its Nramp-like domain and impeding protein ubiquitination.

In addition to OsEIN2, we also investigated the effects of MHZ3 on the protein levels of other ER-localized ethylene signaling components, including OsETR2, OsERS2, and OsCTR2 (32). Transient expression of Myc- or GFP-tagged OsETR2, OsERS2, or OsCTR2 in rice protoplasts from WT, *mhz3*, and *MHZ3*-OX seedlings revealed that the protein levels of the ethylene receptors and OsCTR2 were unaffected by MHZ3 (*SI Appendix*, Fig. S14).

Discussion

In this study, we identified a modulator of OsEIN2 function. Interaction with MHZ3 is required for the proper accumulation of OsEIN2 protein. MHZ3 stabilizes OsEIN2 likely by binding to its Nramp-like domain and impeding protein ubiquitination, avoiding proteasome-mediated protein degradation. Since the MHZ3 sequence is conserved from algae to land plants, the *mhl1 mhl2* double mutant of *Arabidopsis* homologous genes exhibits similar ethylene-insensitive phenotypes; and since the known ethylene signaling components are functionally conserved between lower and higher plants (33), the potential regulatory mechanism of MHZ3 in ethylene signaling may be conserved throughout the plant kingdom.

Multiple lines of genetic evidence suggest that *MHZ3* acts at the *OsEIN2* level in the ethylene signaling pathway. MHZ3 directly interacts with OsEIN2 as demonstrated from the Y2H, Co-IP, and BiFC assays in our study. Protein–protein interaction usually plays a crucial role in regulation of protein stabilization (34–36). Presently, interaction with MHZ3 is required for OsEIN2 accumulation, but not for the ER localization or nuclear translocation of OsEIN2. Our biochemical data show that MHZ3 interacts with OsEIN2 through binding to its N-terminal Nramp-like domain. Without the Nramp-like domain, MHZ3 was unable to stabilize OsEIN2, demonstrating that association of MHZ3 with the

OsEIN2 Nramp-like domain is crucial for OsEIN2 accumulation. Importantly, MHZ3-inhibited OsEIN2 ubiquitination also depends on the presence of the Nramp-like domain. Although extensive studies have demonstrated that the C-terminal domain of EIN2 can be cleaved and translocated into the nucleus and P-body to activate downstream signaling (7, 13, 15, 16, 19, 20), the function of its N-terminal Nramp-like domain has long been unknown. Expression of the EIN2 C-terminal domain in *ein2-5* was unable to restore the triple response, suggesting that the Nramp-like domain is essential for triggering ethylene responses in *Arabidopsis* etiolated seedlings (7). Our findings provide a potential mechanism for how EIN2 works through its Nramp-like transmembrane domain.

Ubiquitination analyses revealed that MHZ3 impedes OsEIN2 ubiquitination likely by binding to its N-terminal Nramp-like domain; however, the underlying mechanism is not clear. One possibility is that the binding of MHZ3 to the OsEIN2 Nramp-like domain may lead to a conformational change in the protein, preventing the binding of ETP1/ETP2-like proteins to OsEIN2 for degradation. Unfortunately, *ETP1/ETP2*-like genes were not identified in the rice genome (37). Thus, identification of the F-box proteins involved in OsEIN2 degradation is necessary to elucidate the mechanisms by which MHZ3 stabilizes OsEIN2. On the other hand, ethylene receptors, CTR1, and EIN2 can form a signaling complex on the ER through protein–protein interactions (38–40). Therefore, as an alternative mode of action, MHZ3 may function as a molecular chaperone involved in the regulation of the signaling complex, although the protein levels of OsETR2, OsERS2, and OsCTR2 were not affected by MHZ3.

It should be noted that although ethylene induces *MHZ3* expression, the fact that the *35S:MHZ3-GFP* transgene can restore normal ethylene response in *mhz3* mutant suggests that the ethylene regulation of *MHZ3* may be dispensable for the function of this gene in the ethylene response. Additionally, *MHZ3* overexpression with massive accumulation of the protein leads to only slight ethylene hypersensitivity. In contrast, mild accumulation of MHZ3 in response to ethylene is sufficient to trigger ethylene response. These facts suggest an alternative mechanism by which MHZ3 may play a housekeeping role rather than a regulatory function in the signaling pathway.

Collectively, we identified the ethylene-induced and ER-localized modulator MHZ3, which interacts with the Nramp-like domain of OsEIN2 and stabilizes the protein to facilitate ethylene signaling. Manipulation of the gene may improve agronomic traits, especially in food crops.

Materials and Methods

Details for plant growth and ethylene-response assay, gene expression analysis, protein fractionation, membrane-based Y2H assay, Co-IP and BiFC assays, and ubiquitination analysis are described in *SI Appendix, SI Materials and Methods*. The primers used in this study are listed in *SI Appendix*, Table S1.

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