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

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The phytohormone ethylene regulates multiple 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 mh3. 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 N-terminal domain. Loss of mh3 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.

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 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 mh3 mutant. MHZ3 encodes a previously uncharacterized ER membrane protein that genetically acts on and physically interacts with OsEIN2. MHZ3 associates with the OsEIN2 N-terminal domain to stabilize the protein by inhibiting ubiquitination and 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.

T he 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 Set/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 to 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 mh3 mutant. MHZ3 encodes a previously uncharacterized ER membrane protein that genetically acts on and physically interacts with OsEIN2. MHZ3 associates with the OsEIN2 N-terminal domain to stabilize the protein by inhibiting ubiquitination and 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 N rampage-like domain.

**Results**

**Phenotypic Analysis and Gene Identification of mh3-1 Mutant**

Five allelic mh3 mutants (mh3-1 to mh3-5) 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. 1A). By contrast, the effects of ethylene on coleoptile and root growth of etiolated rice seedlings are almost completely blocked by the mh3 mutation (Fig. 1A 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 mh3 seedlings (Fig. 1B). These results suggest that mh3 is insensitive to both endogenous and exogenous ethylene. The ethylene responsiveness of mh3 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 mh3 mutant (Fig. 1C).

The MH3 gene was identified by map-based cloning. All mh3 mutants harbored a mutation at the LOC_Os06g02480 locus (Fig. 1D). The mutation sites were further confirmed by PCR-based analysis using cleaved amplified polymorphic sequence (CAPS) or derived CAPS primers (SI Appendix, Fig. S1C). Genetic transformation with the WT genomic DNA fragment rescued the ethylene-insensitive phenotype of mh3-1 (Fig. 1E). The results convincingly demonstrate that MH3 is located at the LOC_Os06g02480 locus. Gene expression and promoter-GUS analyses revealed that MH3 is universally expressed in the rice organs examined from vegetative to reproductive stages (SI Appendix, Fig. S2). The deduced MH3 protein has no domains of known function, except for a signal peptide (SP) and one transmembrane helix (TM) (Fig. 1D). BLAST search and phylogenetic analysis revealed that MH3 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 MH3 is a single-copy gene in the rice genome but has two copies in the Arabidopsis genome. We named these Arabidopsis homologous genes MH3- LIKE1 (MHL1, At1g75140) and MHL2 (At1g19370), which share 40% and 38% identity with MH3, respectively. Although the mhl1 and mhl2 single mutants exhibited WT-like ethylene response likely due to functional redundancy, the mhl1 mh2 double mutant displayed ethylene-insensitive phenotype, indicating that MHL1 and MHL2 are required for ethylene signaling in Arabidopsis (Fig. 1F 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 MH3-like genes may not be as strong as that of EIN2 in ethylene signaling.

**MH3 Is an ER-Localized Glycosylated Membrane Protein.** We generated a specific anti-MH3 antibody for detecting the endogenous protein. Total proteins of WT seedlings were separated into soluble and microsomal fractions by ultracentrifugation. MH3 was mainly detected in the microsomal fraction, indicating that MH3 is a membrane-bound protein (Fig. 2A). The microsomal membranes were further separated into plasma membrane (upper phase) and endomembrane systems (lower phase) by aqueous two-phase partitioning. MH3 was solely detected in the lower phase, indicating an endomembrane localization of MH3 (Fig. 2B). To further determine the subcellular localization, we transiently expressed MH3-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 MH3 is predominantly localized at the ER (Fig. 2C). In addition, we observed fluorescent signals in distinct dots along with the ER networks (Fig. 2C), implying that MH3 may also target to organelles/compartment other than the ER. Its localization outside the ER may suggest that MH3 has additional functions in ethylene signaling and/or in other processes. The MH3 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 MH3 (Fig. 2D).

**MH3 Expression Is Induced by Ethylene, and the Gene Overexpression Confers Ethylene Hypersensitivity.** We examined MH3 gene expression in response to ethylene. The transcript level of MH3 was significantly induced by ethylene treatment in both shoots and roots of etiolated WT seedlings, whereas the induction was largely blocked in Oseinz2-1, Osers29 (a dominant ethylene receptor gain-of-function mutation), and Oseil1 seedlings (Fig. 2E). In Arabidopsis, the transcript level of MHL2 was also significantly induced by ethylene, whereas MHL1 expression was unaffected by ethylene.
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 *Osein2* and *Oseil2* ethylene-receptor loss-of-function mutants was completely abolished by *mhz3* mutation, indicating that MHZ3 may act at or downstream of ethylene receptors (Fig. 3A). To examine the epistatic relationship between MHZ3 and *CTR1*, we constructed the *mhl1 mhl2 ctr1* triple mutant of *Arabidopsis*. The constitutive signaling caused by *ctrl* mutation was fully suppressed by *mhl1 mhl2* double knockout, implying that MHZ3 may act at or downstream of *ctrl* (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. 3B) (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. 3B). 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 N-rramp-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. 3C). However, MHZ3 overexpression in Osein2-2 was unable to suppress the mutant phenotype (Fig. 3D). 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. 3E). 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. 3F). 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. 3F), indicating that MHZ3 is required for ethylene-induced OsEIL1 activity. Considering the fact that ethylene-induced EIN3/EI1 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. SRP014568). 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 N-rramp-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
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. S12A). 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 than that observed in the WT (SI Appendix, Fig. S12B). 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. S12C). 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. 5A, Left). However, in \textit{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. 5A, Left). Similarly, in the OsEIN2-overexpressing line in the WT background, the OsEIN2 level gradually increased upon ethylene treatment, but in the \textit{mhz3} background, OsEIN2 was detected at very low levels, and no detectable accumulation of the protein was observed upon ethylene treatment (Fig. 5A, Right). The results suggest that \textit{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, \textit{Osein2-1}, and \textit{Osein2-2} backgrounds using the transgenic lines in Fig. 3C and D. MHZ3 overexpression markedly elevated OsEIN2 abundance in WT seedlings (Fig. 5B). Similarly, overexpression of MHZ3 in \textit{Osein2-1} and \textit{Osein2-2} backgrounds apparently enhanced accumulation of the Osein2 mutant proteins which were almost undetectable in the single mutants (Fig. 5B). 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. 4D). GFP-tagged full-length OsEIN2 and Osein2-1 and the truncated OsEIN2 C-terminal domain were transiently expressed in rice protoplasts isolated from \textit{mhz3} mutant and \textit{MHZ3-OX} (OX22) seedlings. The full-length OsEIN2 and Osein2-1 proteins obviously accumulated when expressed in OX22 protoplasts compared with those in the \textit{mhz3} mutant (Fig. 5C). This is consistent with the observations in stable transgenic plants (Fig. 5B). By contrast, no significant difference in the protein levels of the truncated OsEIN2 C-terminal domain was detected when expressed in \textit{mhz3} mutant and OX22 backgrounds (Fig. 5C). 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.

**MHZ3 Impedes OsEIN2 Ubiquitination.** To explore the underlying mechanisms by which MHZ3 stabilizes OsEIN2, we treated \textit{mhz3} seedlings with the proteasome inhibitor MG132. The treatment partially restored OsEIN2 accumulation in \textit{mhz3} mutant, suggesting that MHZ3 modulates OsEIN2 accumulation through the proteasomal pathway (Fig. 5D). Next, we examined the ubiquitination of OsEIN2 in different MHZ3 backgrounds using the protoplastic transient expression system. Compared with that in WT protoplasts, the ubiquitination level of OsEIN2 was obviously enhanced in \textit{mhz3} mutant but suppressed in \textit{MHZ3-OX} backgrounds, suggesting that MHZ3 negatively modulates OsEIN2 ubiquitination (Fig. 5E). In comparison with the full-length OsEIN2, no significant difference in the ubiquitination level of the OsEIN2 C-terminal domain was detected in WT, \textit{mhz3}, and \textit{MHZ3-OX} backgrounds (Fig. 5F), 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, \textit{mhz3}, and \textit{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 \textit{mhl1 mhl2} double mutant of \textit{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 ein2-2 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, OsEERS2, and OsCTR2 were not affected by MHZ3.

It should be noted that although ethylene induces MHZ3 expression, the fact that the 33S:MHZ3-GFP transgene can restore normal ethylene response in mh3 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, 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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