Supporting Information

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

Ma et al.

SI Materials and Methods

Plant materials and growth conditions. The rice (*Oryza sativa* L.) mutants *mhz3*, *mhz7-1*/*Osein2-1*, *mhz7-2*/*Osein2-2* and *mhz6*/*Oseil1* were previously identified in our laboratory (1, 2). The root-specific ethylene-insensitive mutant *mhz12*/*Osers2*^{d} was identified in our genetic screen, which harbors a dominant gain-of-function mutation A32V that is equivalent to *Arabidopsis etr1-3* (3). T-DNA insertion knockout mutants of *Osers2* and *Osetr2* were obtained from the POSTECH Biotech Center and identified previously (4). For material propagation and crossing, rice plants were grown in the Experimental Farm Stations of the Institute of Genetics and Developmental Biology in Beijing from May to October and in Hainan from November to next April. Ethylene treatment of rice seedlings was performed as previously described (5). Briefly, rice seedlings were grown on stainless steel sieves that were placed in 5.5-L air-tight plastic containers supplied with various concentrations of ethylene. The seedlings were incubated for 2 to 3 days at 28 °C in the dark. At least 30 seedlings were measured for root and coleoptile lengths. For qRT-PCR analysis of ethylene-inducible genes in WT and *mhz3*, 2-d-old etiolated seedlings were treated with 10 ppm ethylene for 8h. For qRT-PCR analysis of *MHZ3* in the shoots and roots of WT and *Osein2-1* and in the roots of WT, *Osers2*^{d} and *Oseil1*, 2-d-old etiolated seedlings were treated with 10 ppm ethylene for 0-12 h and 12 h, respectively. For qRT-PCR analysis of Arabidopsis *MHL1/2*, 5-d-old etiolated seedlings of Col-0 and *ein2-5* were treated with 10 ppm ethylene for 0-12 h. For immunoblot analysis of MHZ3 level in response to ethylene, 2-d-old etiolated rice seedlings were treated with 10 ppm ethylene for 0-24 h for WT and *Osein2-1* and 24 h for WT, *Osers2*^{d} and *Oseil1*. For MG132 treatment, 3-d-old etiolated rice seedlings were treated with 50 μM MG132 (474790, Calbiochem) or 0.1% DMSO (mock) dissolved in deionized water for 4 h at 28°C in the dark. For triple response assay of *Arabidopsis*, the seeds were surface-sterilized and sown on 1/2 MS (1% sucrose, 0.8% agar, pH 5.7). After 3 days of stratification at 4 °C, the plates were incubated in the dark at 22 °C for 4 days in the absence (air) or presence of 10 ppm ethylene. Hypocotyl length and root length were measured using ImageJ software (National Institutes of Health). Angles of apical hooks were assayed as previously described (6).

Map-Based Cloning of *MHZ3* Gene. Previous study revealed that *mhz3* is a recessive mutation at a single nuclear locus (1). Using F2 mapping populations derived from crosses between *mhz3-1* and indica varieties 93–11, MH63, TN1 and ZF802, *mhz3* locus was mapped to chromosome 6 within a 1.18Mb region between Idl6-0.72 and Idl6-1.9 markers (Table S1). The candidate gene was determined by DNA sequencing of all the genes in this region. The mutation sites in *mhz3* allelic mutants were identified by DNA sequencing and confirmed by PCR-based analysis using CAPS or dCAPS primers (Table S1).

Epistasis analysis. *Osetr2* and *Osers2* mutants are in Dongjin (DJ) background, and *mhz3* in Nipponbare (Nip) background. *Osers2 mhz3* and *Osetr2 mhz3* double mutants were generated by crossing the corresponding single mutants. WT (Nipponbare) rice plants were transformed with *35S*:*OsEIN2* transgene (*35S*:*OsEIN2*/WT), and *35S*:*OsEIN2*/*mhz3* was generated by crossing the *mhz3* mutant with the *35S*:*OsEIN2*/WT plant. The WT, *Osein2-1*, *Osein2-2* and *Oseil1* rice plants were transformed with *35S:MHZ3* transgene and the transgenic lines in different backgrounds with similar expression levels of *MHZ3* gene were used for ethylene-response analysis. The WT and *mhz3* plants were transformed with *35S:OsEIL1* transgene and the transgenic lines in different backgrounds with similar expression levels of *OsEIL1* gene were used for ethylene-response analysis.

Gene expression analyses. Total RNAs were isolated from rice seedlings using a TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNAs were synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). qRT-PCR analyses were performed with three biological replicates and two technical replicates. GUS staining assay and RNA-seq analysis were performed as described previously (7).

Plasmid construction and rice transformation. To generate *MHZ3*-complementation construct, a 6400-bp genomic DNA of *MHZ3* (2509 bp before the start codon and 1965 bp after the stop codon) was PCR-amplified and cloned into *Hind III/Bsa I* digested pCAMBIA2300 vector. To construct *MHZ3pro-GUS*, a 2000-bp promoter region was PCR-amplified and cloned into *Hind III/Bam HI* digested pCAMBIA2300-35S-GUS vector to replace the CaMV35S promoter. The *MHZ3* coding sequence was PCR-amplified and digested by *Xba I/Bsa I* and cloned into *Xba I/Sal I* digested pCAMBIA2300-35S vector to generate 35S:*MHZ3* construct for *MHZ3* overexpression. The *GFP* fragment was cut from a pUC18-based vector and inserted into *Sal I/Pst I*-digested pCAMBIA2300-35S vector to generate pCAMBIA2300-35S-GFP plasmid. To construct *35S:MHZ3-GFP*, *MHZ3* coding sequence was PCR-amplified and cloned into *Kpn I/Xba I*-digested pCAMBIA2300-35S-GFP vector. The coding sequences of *OsEIN2* and *OsEIN2-C* (489-1281 aa) were PCR-amplified and cloned into *Sma I/Sal I* digested

pCAMBIA2300-35S-GFP vector to generate *35S:OsEIN2-GFP* and *35S:OsEIN2-C-GFP* constructs. The coding sequence of *OsEIN2-1* was PCR-amplified from *Osein2-1* mutant and subcloned into pENTR/D-TOPO vector (Invitrogen) then cloned into pGWB405 (Invitrogen) vector by homologous recombination. For interaction domain mapping assays, the full length or truncated versions of *MHZ3* and *OsEIN2* coding sequences were PCR-amplified and cloned to *Bam HI*-digested pCambia2300-35S-cYFP-Myc and pCambia2300-35S-nYFP-FLAG by homologous recombination, respectively. To generate *OsERS2-Myc*, the coding sequence was PCR-amplified and cloned into *Bam HI/Sal I*-digested pCAMBIA1300-35S-10xMyc vector. To generate *OsETR2-Myc*, the coding sequence was PCR-amplified and cloned into *Xba I/Sal I* digested pCAMBIA1300-35S-10x Myc vector. To generate *OsCTR2-GFP*, the coding sequence of *OsCTR2* (Loc_Os02g32610) was PCR-amplified and cloned into *Bam HI/Sal I*-digested pUC18-35S-GFP vector through homologous recombination. The primers used for the plasmid constructions are listed in Table S1. *Agrobacterium*-mediated rice transformation was performed as described previously (7).

Antibody generation and immunoblot analysis. For preparation of MHZ3 antibody, the cDNA fragment encoding 21 to 270 amino acids of MHZ3 was cloned into pQE30 Xa vector (Qiagen) and expressed in *E. coli* M15. The recombinant protein was purified using HisTrap-HP column (Amerhsam) under denaturing conditions and used to raise polyclonal antiserum in mice. For OsEIN2 antibody generation, a synthetic KLH-conjugated peptide (PNILESDNKPLGGNNPS) was used as an immunogen to raise polyclonal antiserum in mice. For immunoblot analysis, proteins were heated with SDS-PAGE loading buffer at 65°C for 5 min and separated using SDS-PAGE. Primary antibody dilutions were in PBS containing 3% milk and 0.1% Tween 20. For OsEIN2 detection, the primary antibody was diluted in Immunoreaction Enhancer Solution I (Toyobo). The primary antibodies used include: anti-MHZ3 (1:2,000), anti-OsEIN2 (1:10,000), anti-GFP (7G9) (1:5,000; M2004, Abmart), anti-H^+-ATPase (1:2,000; PM marker; AS07 260, Agrisera), anti-BiP (1:5,000; ER marker; AS09 481, Agrisera), anti-UGPase (1:5,000; cytoplasm marker; AS05 086, Agrisera), anti-histone H3 (1:10,000; nuclear marker; AS10 710, Agrisera), anti-c-Myc (9E10) (1:1000; sc-40, Santa Cruz), anti-FLAG (FLA-1) (1:10,000; M185-3, MBL). Secondary goat anti-rabbit or anti-mouse-IgG-horseradish peroxidase (M210011, M210021, Abmart) antibodies were used at 1:10,000 dilutions in PBS. The signals were detected by chemiluminescence method using SuperSignal West Pico kit (34080, Thermo Scientific) or Smart-ECL Super kit (S32500-1, Smart-Lifesciences). When needed, the signal intensities were quantified by ImageJ software with default parameters (National Institutes of Health).

Total and membrane protein isolation. To isolate microsomal membrane proteins, five grams of etiolated rice seedlings were ground in liquid nitrogen to a fine powder and dissolved in 10 mL extraction buffer [100 mM Tris-HCl (pH 8.0), 150 mM KCl, 5 mM EDTA, 10% Glycerol (v/v), 3.3 mM dithiothreitol (DTT), 0.6% (w/v) polyvinylpyrrolidone (PVPP), 1% (v/v) protease inhibitor cocktail (Sigma)]. The homogenates were filtered through two layers of miracloth (Calbiochem) and centrifuged at 10,000 g for 5 min at 4°C twice to remove debris. The supernatants were recovered and centrifuged at 100,000 g for 60 min at 4°C to pellet the microsomal membranes. The membrane pellets were washed three times with extraction buffer and dissolved in 200 µL extraction buffer containing 1% (v/v) Triton X-100 and 0.1% (w/v) SDS by incubating on ice for 30 min. For PNGase F treatment, the membrane pellets (30 µg) from WT seedlings were denatured and then incubated at 37ºC for 2 h in the presence of 1/10 volume of PNGase F (P0704S, NEB) or 50% glycerol (mock).

For analyses of OsEIN2 protein, total proteins were isolated as described by Li *et al.* (8) with minor modifications. Etiolated rice seedlings were ground in liquid nitrogen and homogenized in 1/2 volume (0.5 mL/gram) of extraction buffer [50mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5% β-mercaptoethanol, 10% (v/v) Glycerol, 0.5% (v/v) NP-40, 2 mM PMSF, 2x complete protease inhibitor (Roche)]. The homogenates were centrifuged at 12,000 g for 10 min at 4°C. The supernatants were recovered and frozen in liquid nitrogen until use.

Nuclear protein isolation. The nuclei isolation was performed using the CelLytic PN Plant Isolation/Extraction Kit (CELLYTPN1, Sigma-Aldrich). Five grams of shoot tissues of 3-d-old etiolated rice seedlings were ground in liquid nitrogen, homogenized in 15 ml nuclei isolation buffer (NIB), and passed through a 100-mesh filter plus 1-layer miracloth. The lysates (total protein) were separated into cytoplasmic fractions and nuclear pellets by centrifugation at 1,260 g for 20 min. The cell membranes were lysed with NIBA containing 0.3% Triton X-100. Crude nuclei were washed 8-10 times with NIBA. Semi-pure preparation of nuclei was performed with a cushion of 2.3 M sucrose. Nuclear proteins were extracted from the nuclei suspended in NIBA-diluted 1x SDS-PAGE loading buffer.

Two-phase partitioning. Twenty grams of 3-d-old etiolated rice seedlings of WT were ground in liquid nitrogen to a fine powder and dissolved in 40 mL of homogenization buffer [50 mM HEPES-KOH (pH 7.5), 0.5 M sucrose; 5 mM ascorbic acid; 1 mM DTT, 0.6% (w/v) PVPP, 1x complete protease inhibitor (Roche)]. Aqueous two-phase partitioning was performed using a Dextran T500/PEG3350 phase system as previously described (9).

Subcellular localization. For analyses on subcellular localization of MHZ3, the plasmid DNAs of 35S:*MHZ3-GFP*, 35S:*mCherry-HDEL* and 35S:*GFP* were transiently expressed in tobacco (*Nicotiana benthamiana*) leaf epidermal cells by microprojectile bombardment as previously described (9). The images were taken using a confocal microscopy (Zeiss LSM 710). Excitation/emission wavelengths were set at 488 nm/500–530 nm for GFP, 561 nm/582-654 nm for RFP, and 561 nm/582-639 nm for mCherry.

Membrane-based Y2H assay. *MHZ3*-coding sequence was cloned into the bait vector pBT3-SUC (MHZ3-Cub) and *OsEIN2* into

the prey vector pPR3-N (NubG-OsEIN2) from the DUAL membrane starter kit SUC (Dualsystem Biotech) following the manufacturer's instructions. Yeast strain NMY32 cells were cotransformed with the bait and prey constructs. To detect self-activation, the empty bait and prey vectors were cotransformed with the NubG-OsEIN2 and MHZ3-Cub constructs, respectively. The wild type N-terminal half of ubiquitin NubI (pOst1-NubI) was cotransformed with MHZ3-Cub to detect functional expression of MHZ3 protein. Combination of pTSU2-APP and pNubG-Fe65 (provided in the kit) was used as a positive control for protein-protein interaction. Positive transformants were selected on SD-Trp-Leu medium, and protein-protein interactions were detected on SD-Trp-Leu-His-Ade medium. The growth of yeast colonies was investigated after 4 d incubation at 30°C.

BiFC assays. For interaction domain-mapping studies, the full length or truncated versions of *MHZ3* and *OsEIN2* coding sequences were fused to the C-terminal half (cYFP) and N-terminal half of YFP (nYFP), respectively. The cYFP and nYFP were tagged with Myc and FLAG respectively. BiFC constructs were cotransformed into rice shoot protoplasts isolated from 7-d-old etiolated seedlings of *mhz3 Osein2* double mutant as previously described (10). The protoplasts were incubated at 28°C for 12-16 h in the dark. YFP fluorescence was detected using a confocal microscopy (Leica TCS SP5) with 514 nm/525-565 nm excitation/emission wavelengths. For BiFC assays in tobacco leaf cells, plasmids were introduced into *Agrobacterium* strain EHA105 by electroporation. Tobacco (*Nicotiana benthamiana*) leaves of 4-week-old plants were infiltrated with different combinations of *Agrobacterium.* The samples were observed 48 h after *Agrobacterium* infiltration.

Co-IP assays. For coimmunoprecipitation of MHZ3 with OsEIN2 *in planta*, transgenic rice seedlings stably expressing *35S:OsEIN2-GFP* or *35S:GFP* (as negative control) were grown in the presence of 10 ppm ethylene for 3 days in the dark. One gram of shoot tissues was ground in liquid nitrogen and homogenized 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 (Sigma)]. The samples were incubated on ice for 15 min to dissolve the ER membranes and then centrifuged at 20,000 g for 5 min at 4°C twice. The supernatants were incubated with 30 μL of GFP-Trap_A (gta-20, Chromotek) beads by tumbling end-over-end for 1 h at 4°C. The beads were washed six times with washing buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1x complete protease inhibitor (Roche)] by tumbling end-over-end for 5 min at 4°C and then centrifuging at 2,500 g for 2 min at 4°C. The beads were resuspended with 50 μL 2x SDS-PAGE loading buffer and heated at 65°C for 5 min. The eluted immunoprecipitates were immunoblotted with anti-GFP, anti-MHZ3, and anti-BiP antibodies.

For Co-IP assays using protoplast transient expression system, the constructs in various combinations were cotransformed into rice shoot protoplasts. The protoplasts were incubated at 28°C for 16 h in the dark. Total proteins were immunoprecipitated with anti-c-Myc affinity gel (E6654, Sigma-Aldrich) according to the manufacturer's instructions or with GFP-Trap_A beads as described above. The IP and washing buffers for anti-c-Myc IP were same as that for anti-GFP IP.

In vivo ubiquitination analysis. To detect the ubiquitination states of OsEIN2 in different genetic background of *MHZ3*, *35S:OsEIN2-GFP* or *35S:OsEIN2-C-GFP* combined with *35S:FLAG-Ub* (11) were cotransformed into etiolated shoot protoplasts of WT, *mhz3* and *MHZ3*-OX22 seedlings. The protoplasts were incubated for 16 h in the presence of 3 μM MG132. Total proteins were immunoprecipitated with GFP-Trap_A beads as described above and immunoblotted with anti-FLAG, anti-GFP, anti-MHZ3 and anti-BiP antibodies.

SI References

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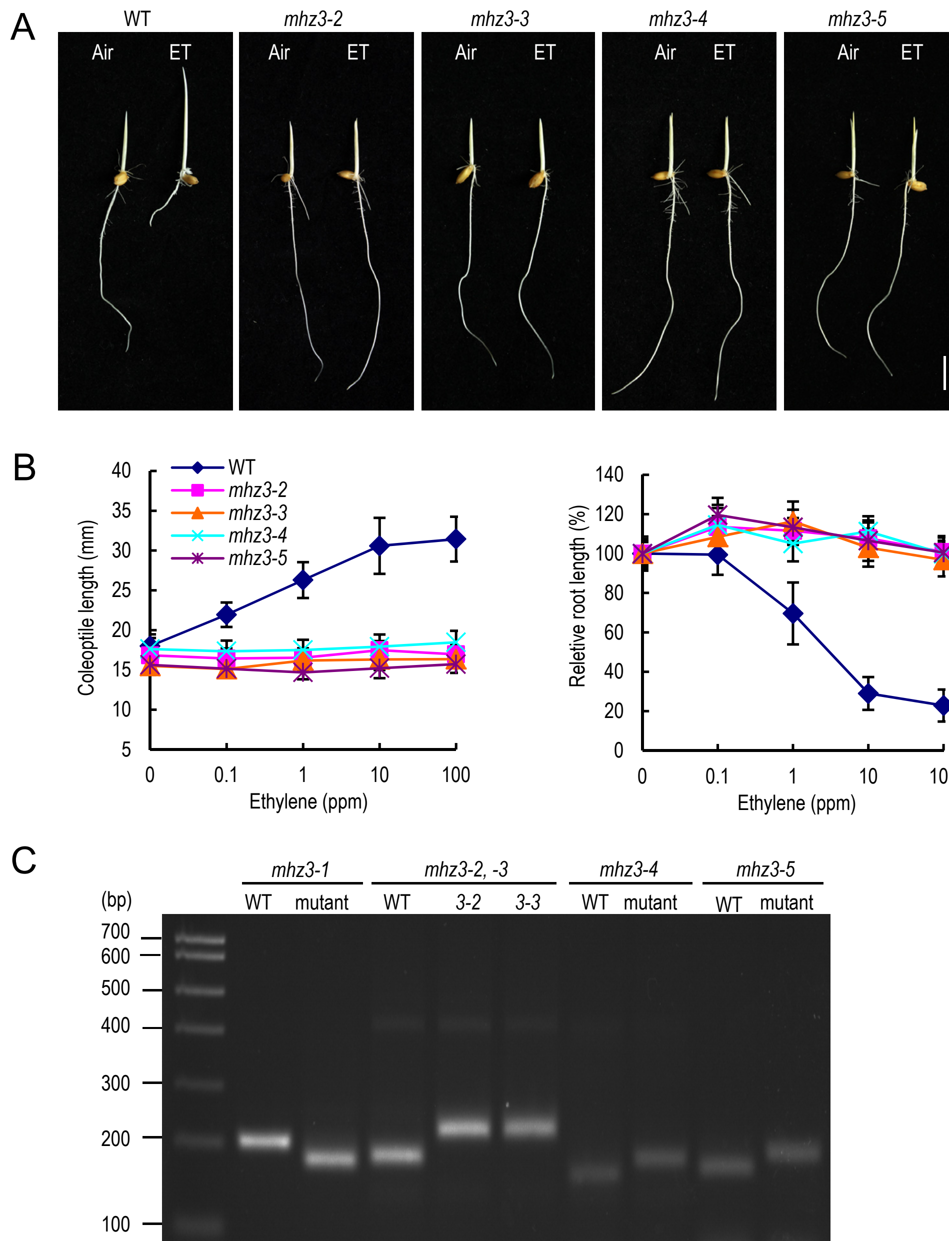

Fig. S1. Identification of *mhz3* allelic mutants. (*A*) Ethylene-response phenotypes of wild-type (WT) and *mhz3-2,-3,-4,-5* etiolated seedlings grown in the air or 10 ppm ethylene for 3 days. Scale bar=10 mm. (*B*) Ethylene dose-response curves for coleoptile length (left) and relative root length (right) in WT and *mhz3* seedlings. Rice seedlings were grown in the dark for 3 days in the presence of various concentrations of ethylene. Means ± SD (n>30). (*C*) Confirmation of *mhz3* mutation sites by PCR-based analysis using genomic DNA as templates. The mutation in *mhz3-1* was confirmed using a pair of dCAPS primers. The PCR products were digested with *Hind* III, resulting in a 27bp-deletion in the mutant but not in WT. The point mutations in *mhz3-2,-3* were confirmed using a pair of CAPS primers. The PCR products were digested with *Nco* I, resulting in a 26bp-deletion in WT but not in the mutants. The mutation in *mhz3-4* was confirmed using a pair of dCAPS primers. The PCR products were digested with *Bam* HI, resulting in a 22bp-deletion in WT but not in the mutant. The point mutation in *mhz3-5* were confirmed using a pair of dCAPS primers. The PCR products were digested with *Nco* I, resulting in a 22bp-deletion in WT but not in the mutant.

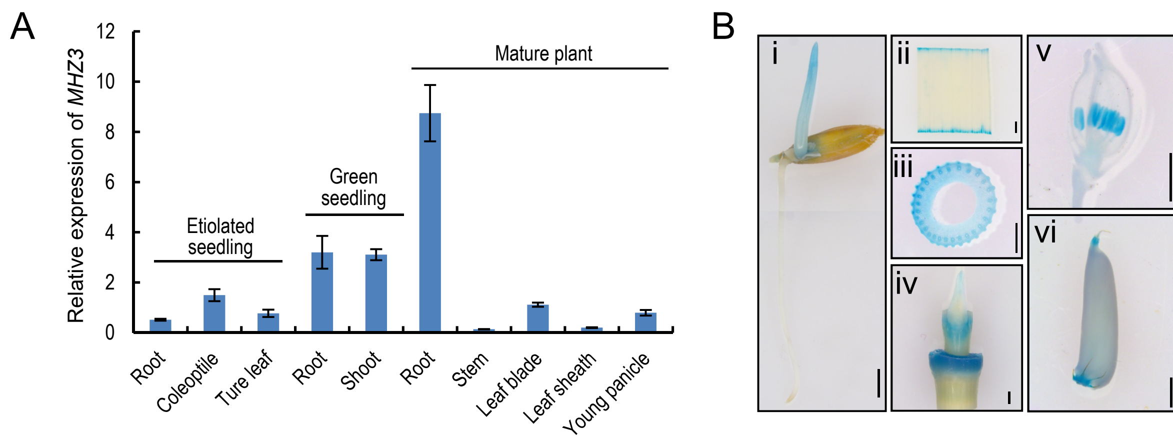

Fig. S2. Spatiotemporal expression analysis of *MHZ3* gene. (*A*) *MHZ3* expression in different rice organs revealed by qRT-PCR analysis. (*B*) Tissue-specific expression of *MHZ3* revealed by promoter-GUS analysis. Transgenic plants expressing *MHZ3*pro::*GUS* were used for analysis. Rice organs/tissues were stained for GUS for 48 h. At least 10 samples for each organ/tissue were observed and representative ones are presented. (i) 3 day-old etiolated seedling. (ii) GUS staining in cutting edge of mature leaf blade. (iii) GUS staining in young stem. (iv) GUS staining in stem node and the base of axillary bud. (v) GUS staining in the anthers of young flower. (vi) GUS staining in the top and bottom of an ovary. Bars=2mm.

Signal peptide

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MHZ3                   1 : MAHHVAP----LLLLLT---LAAVAAASEEAAA-------------------------------- :  27
Apple                  1 : MNSL-K--GKLLLLCFL---FILSSPSPVSKLFVQ------------------------------ :  29
Arabidopsis            1 : MADSQNGKSAFFFF-FVSLILLFLSPSYS-DVTAS------------------------------ :  33
Barley                 1 : MAHLAPAPLLLLLFLLV---AVAASGEEAAGSAR------------------------------- :  31
Brachypodium           1 : MAHRA-----LLFFLLL---AAAAAGSEAEEAEV------------------------------- :  26
Cassava                1 : MANTHK--GKYLSFLYLL--FILAS----ISVNFSSSIATV------------------------ :  33
Castor bean            1 : MASTHK--GKLFSFLYFI--FILVA----VFINYSRSVPIQ------------------------ :  33
Citrus                 1 : MANFHK--GKFFLLYLL---FIFAFSPNDKILVSS----IQ------------------------ :  32
Cocoa bean             1 : MANPLQ--GKFLLVLFL---FMFTSPPLSVVFVNANSVSIQ------------------------ :  36
Cotton                 1 : MANPHK--SRFFLFFFLICHFIFISTPFPTVLANS-----K------------------------ :  34
Cucumber               1 : MAIERK--GRFFFIFFL---FIFAFPHVS-----------R------------------------ :  25
Eucalyptus             1 : MGIRDN--GKFLLLYLL---LVFSHVPGFWVFAD------------------------------- :  29
Eutrema salsugineum    1 : MADSRNGKSFFFCFSFFVSLLLFLSPDISPVVAAS------------------------------ :  35
Flax                   1 : MEDTQKT-GKLFFLLLTILILILLSSPHLTYSSST------------------------------ :  34
Grapevine              1 : MANHHK--GKFFWLYLL---FLFASPPAVRIFINCSPD--------------------------- :  33
Maize                  1 : MPHRVAA----LLLLLP---LAAASEEAP------------------------------------ :  22
Micromonas             1 : MRRRFTLLALLLAILVIVSSRASADETHAPGLDDADDASSS------------------------ :  41
Millet                 1 : MPHRVAA----LLLLLP---LAAASEEAAAAPVA------------------------------- :  27
Moss                   1 : MGKHRADIDMPLRCFMLSICLSLIWSQVLAAITLTDEDWEAGLGADMCPEKTDIPVDSVFPSRGV : 65
Peach                  1 : MSCSIK--GKLILLYFL---FIFSSPSSVSKLFVK------------------------------ :  30
Poplar                 1 : MANTHQ--GKLFFFLYLL--FISCSPSRILADSSSVSTQQE------------------------ :  37
Sorghum                1 : MPHRVAA----LLLLLP---LAAASEEAPVLAPA------------------------------- :  27
Soybean                1 : MAWSHK--GKFFIFSLL---FFFSSLHISSSPLDPESCSIQ------------------------ :  36
Strawberry             1 : MAITAK--GKLILISFL---FVFSS----SKLVLV------------------------------ :  26
Tomato                 1 : MTSLYK--GKIFLFSLL---LLFHVSWVFRAFAEVVIEE-------------------------- :  34
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mhz3-4 NxS/T

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MHZ3                 239 : HHAGRARYVLSCDSGGRIRVFTENGTLYG-TAIASSTP-----------------LAFVK----Q : 281
Apple                261 : HHVGRLNYVLASDVSGKLSVYREDGSVHG-STMPSSRP-----------------LAFLR----Q : 303
Arabidopsis          244 : HHVGRVRYILATDLSGKLTVFTENRTVYG-SVSPTSRP-----------------LVFLK----Q : 286
Barley               267 : HHAGRSRYVLSCDAGRRIRVFTENGTLYG-TAIASSTP-----------------LAFVK----Q : 309
Brachypodium         250 : HHAGRSRYVLSCDAGRRIRVFTENGTLYG-TAIASSTP-----------------LAFVK----Q : 292
Cassava              267 : HHVGRSRYILSSDVGGKIRVFRENGTVHG-SAMPTSRP-----------------LAFLK----Q : 309
Castor bean          276 : HHVGRSRYILSSDVGGKIRILRENGTVHG-SVIPTSRP-----------------IAFLK----Q : 318
Citrus               280 : HHIGRMRYILSADASGKIRVFKENGMVHGTAAMLSSKP-----------------LVFLK----Q : 323
Cocoa bean           272 : HYVGRMRYILSTDLSGKIRVFRENGSLYG-SAMPTSRP-----------------LVFLK----Q : 314
Cotton               241 : HHVGRMRYILCTDLSGKIQVFREEGTLYG-SAMPRSRP-----------------LVFLK----Q : 283
Cucumber             272 : HHVGRTRYILSSDFGGKIKVFREDGTVYG-SVMPTSRP-----------------IAFLK----Q : 314
Eucalyptus           271 : HHVGRMRYIISTDISGRINILKEDGTAYGSVIEPSSRP-----------------LAFLK----Q : 314
Eutrema salsugineum  255 : HHVGRVRYILATDLSGKLTVFTENRTVYG-SVTPTSRP-----------------LVFLK----Q : 297
Flax                 244 : HHVGRLRYILSSAASGKIRVFKEDGKLHG-SVTPSSRP-----------------LAFLK----Q : 286
Grapevine            238 : HHIGRMRYILSTNIGGEIRVFRENGSVHG-SAKPMSRP-----------------LVFLK----Q : 280
Maize                230 : HHAGRARYVLSCDAGGRIRVFTENGTLYG-TAIASSTP-----------------LAFVK----Q : 272
Micromonas           420 : FRIRDVRYVAVADASGKVAVFRVPTGGYNGSGARGTHPNQWRLHLHSVHRSVDGVVSFRQSP--H : 482
Millet               236 : HHAGRARYVLSCDAGGRIRVFTENGTLYG-TAISSSTP-----------------LAFVK----Q : 278
Moss                 374 : YRVGKMRYVLVADSTGKMQVFRENGTLFG-AADSSSRP-----------------LAFLRTPNTQ : 420
Peach                262 : HHVGRVSYILASDVSGKITVFRENGSVHG-STMPSSRP-----------------LAFLK----Q : 304
Poplar               279 : HHVGRSRYILSSDVSGVIRVFRENGTVHG-SAIPTSRP-----------------LAFLK----Q : 321
Sorghum              240 : HHAGRARYVLSCDAGGRIRVFTENGTLYG-TAIASSTP-----------------LAFVK----Q : 282
Soybean              251 : HYVGRMKYILSADTSGKIRVFKENGSLHG-SATPSSRP-----------------LVFLK----Q : 293
Strawberry           243 : HHLGRMSYILASDVSGKITVYKENGAVHG-STMPSSRP-----------------LAFLK----Q : 285
Tomato               265 : HHVGRNRYILSTDSGGKLWVFRENGTVYG-VTTPKSRP-----------------LAFLK----Q : 307
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NxS/T mhz3-5

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MHZ3                 282 : RLLFLTEAGAASLDLRS-MSVRETPCEGLAEALNGSLPKAYSFDPSERFKAYGFTDAGDLVHVLL : 345
Apple                304 : RLLFLTETGAGSLDLKT-MKVRESECEG----LNRSHSRYYVFDATERSKAYGFTSEGDLIHVLL : 363
Arabidopsis          287 : RLLFLTETGAGSLDLRS-MKIRESECEG----LNHSLARSYVFDASERAKAYGFTSEGEIIHVLL : 346
Barley               310 : RLLFLTEAGAASLDLRS-MTVRETPCEGLAEALNGSRPRSYSFDPSERFKAYGFTDAGDLVHVLL : 373
Brachypodium         293 : RLLFLTEAGAASLDLGS-MTVRETPCEGLAEALNGSRPKAYSFDPSERFKAYGFTDAGDLVHLLL : 356
Cassava              310 : RLLFLTESGAGSLDLRS-MKVRESECEG----LNHTLVRNYVFDATERSKAYGFTSEGDLIHVLL : 369
Castor bean          319 : RLLFLTESGAGSLDLRN-MKVRESDCEG----LNHSLVRNYVFDATERSKAYGFTSEGNLIHVVL : 378
Citrus               324 : RLLFLTECGAGSLDLRT-MKLRETECEG----LNNSLVRNYVFDATERSKAYGYTSEGDLIHVLL : 383
Cocoa bean           315 : RLLFLTQTGAGSLDLRS-MKIKESECEG----LNHSLALNYVFDPTERSKAYGCTSDGDLIHVLL : 374
Cotton               284 : RLLFLTETGAGSLDLRN-MRIKESECEG----LNHSLARNYVFDATERSKAYGFTSDGDLIHVLL : 343
Cucumber             315 : RLLFLTESGAGSLDLRS-MKLRESECEG----LNHSLARNYVFDAMERSKAYGVTSDGDLIHVLL : 374
Eucalyptus           315 : RLLFLTEKGAGSLDLRS-MKLRESECEG----LNHSLARSYVFDATERSKAYGYTSEGDLIHVSL : 374
Eutrema salsugineum  298 : RLLFLTETGAGSLDLRS-MKIRESECDG----LNHSLARAYVFDSSERSKAYGFTSEGEVIHVLL : 357
Flax                 287 : RLLFLTESGAGSLDLRS-MKIRESDCEG----LNHSLVRNYVFDVTERSKAYGFTSDGHLIHVLL : 346
Grapevine            281 : RLLFLTENGAGSLDLKT-MKVRESECEG----MNHSIAKNYVFDAAERSKAYGFTSDGDLFHVFL : 340
Maize                273 : RLLFLTEAGAASLDLRS-MSVRETPCEGLAEMLNGTSVKAYSFDPSERFKAYGFTEAGDLVHVLL : 336
Micromonas           483 : YVAWVGSRGAGAADVAATLEVLHKPCHN----LNGTHVQRLKFDVAASGRFVGVGVGGELLTGFV : 543
Millet               279 : RLLFLTEDGAASLDLRS-MSVRETPCEGLAEALNGTSVKAYSFDPSERFKAYGFTEAGDLVHVLL : 342
Moss                 421 : RLLFLTKTGGGSLDLRT-MTVRSCPCDG----LNGSTVVAYAFDAAGRSRAYGVTEEGDLVYVIL : 480
Peach                305 : RLLFLTETGAGSLDLRN-MKVRESECEG----LNRSLSRYYVFDATERSKAYGFTSEGDLIHVLL : 364
Poplar               322 : RLLFLTESGAGSIDLRS-MKVRESECEG----LNQTLARNYVFDATERSKAYGFTSGGDLINVLL : 381
Sorghum              283 : RLLFLTEAGAASLDLRS-MSVRETPCEGLAEALNGTSVKAYSFDPSERFKAYGFTEAGDLVHVLL : 346
Soybean              294 : RLMFLTETGAGSLDLRG-MKIRESECEG----LNHSVARTYVFDATERSKAYGFTSDGDLIYVLL : 353
Strawberry           286 : RLLFLTETGAGSLDLRT-MKIRESECEG----LNHSLSRYYVFDATERSKAYGFTSEGELIHVVL : 345
Tomato               308 : RLLFLTETGAGSLDLRT-MKIRESECEG----LNNSIAKSYVFDATERSKAYGFTSDGDLIHVLL : 367
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                                                                  NxS/T
MHZ3                 346 : LGDIASLKCRVRAIKKAE-IDNP---VAIQTIKGYLLVASQDKILVYNTSTQYYGRVG------A : 400
Apple                364 : LGDIMNFKCRVRSKRKFE-IDEP---LAFQAIKGCLLIVSGEKVFVYNVSTQHYVRVG------A : 418
Arabidopsis          347 : LGDIMNFKCRVRSKKKVQ-MEEP---VALQAIKGYLLIVNQEKVFVYNVSTQHYVRTT------G : 401
Barley               374 : MGDVANLKCRVRAVKKAE-VDSP---VAIQTIKGYLLVASQDKIMVYNTSSQYYGRVG------A : 428
Brachypodium         357 : LGDVASLKCRVRAVKKAE-ADSP---VAIQTIKGYLLVASQDKIMVYNTSSQYYGRVG------A : 411
Cassava              370 : LGDVMNFKCRVRSKRKFD-MDEP---IAMQAIKGYLLVVNEEKIFAYNVSTQHYVRVG------G : 424
Castor bean          379 : VGDIMNFKCRVRSKRKFD-MDEP---LTLQAIKGYLLAVNAEKVFAYNVSTQHYVRVG------G : 433
Citrus               384 : LGDVTNFKCRVRSKRKFD-MSEP---LAFQAIKGYLLVVCEEKIFVYNVSAQHYVRSG------G : 438
Cocoa bean           375 : LGDIMNFKCRVRSKKKLE-LRQP---LAFQAIKGYLLIVDQEKVFVYNVSTLHYVRAG------S : 429
Cotton               344 : LGDIMNFKCRVRSKKKLE-INEP---LAFQAIKGYLIIVNTEKVFVFNVSTPHYVRAG------V : 398
Cucumber             375 : LGDIMNFKCRVRSKRKFE-LDEP---LAFQTIKGYLLVTSNEKVHVFNVSSQHYVRVG------A : 429
Eucalyptus           375 : LGDVVNFKCRVRYKKKSE-IEEP---LALETIKGYLLVVNQEKVFVYNVSSHHYVRVG------V : 429
Eutrema salsugineum  358 : LGDIMNFKCRVRSKKKVQ-MEEP---VALQAIKGYLLVVSQEKVFVYNVSTQHYVRTT------G : 412
Flax                 347 : LGDAMNFKCRVRSKKKPDKIDEPP-VAAMQAIKGYLLLVSEESVFVYNVSSHHYVRVG------G : 404
Grapevine            341 : LGDIVNFKCRARSKRRFD-MGEP---LAFQAIKGYLLIVNEEKVFVYNVSSQHYGRVG------G : 395
Maize                337 : LGDVSSLKCRVRSVKKSE-MDNP---VAIQTIKGYLLVASHDKILVFNTSSQYYGRVG------A : 391
Micromonas           544 : NVDANRAMCVVRSVTRPRGLSSLTPDVSLAAIKGYAFAANPYEVSVLNTT-----VVGK----KP : 599
Millet               343 : LGDVSSLKCRVRAVKKAE-IDSP---VAIQTVKGYLLVASHDKILVYNTSSQYYGRVG------A : 397
Moss                 481 : SGDTLHFECHARTTKRKLDVEGP---VSLHGIKGYLVVATPKNVFVYNTTLQLGFSYANIRGSAG : 542
Peach                365 : LGDIMNFKCRVRSKRKFE-IDEP---LAFQAIKGCLLIVSGEKVFVYNVSTQHYVRVG------A : 419
Poplar               382 : LGDIMNFKCRVRSKRKFD-MDGP---LALQSIKGYLFVVNEEKVFVYNVSSQHYVRVG------G : 436
Sorghum              347 : LGDVSSLKCRVRAVKKSE-IDNP---VAIQTIKGYLLVASHDKILVFNTSSQYYGRVG------A : 401
Soybean              354 : LGDVMNFKCRVRYKKKFD-VDEP---LALQAIKGYLLIVNPEKVFVYNVSSPHYVRVG------V : 408
Strawberry           346 : LGDVMNFKCRVRSKRKFE-MDEP---LAFQAIKGCLLIVSGEKVFVYNVSSQHYVRVG------A : 400
Tomato               368 : LGDNMNFKCRVRSKRKLE-MAEP---LSFQAIKGYLLVANQDKVSLYNVSSLHYVRSG------G : 422

                                Transmembrane domain
MHZ3                 461 : KP-ESNAVVWSGPALLFLLFLIGIWQVYVKKKDSL-GWT-PEETFNTSVTAPTGSILNHSTSDRA : 522
Apple                479 : KN-EFNTMLWTSPVFFFVLFLFGAWQFFAKKKEALTSWG-PDDPFTSTSATMGAPLGGSNTGDRS : 541
Arabidopsis          467 : KA-EFNTMLWSSPVFFFILFLFGAWHFFSKKKESLTAWG-PDDPFSSTTMSSSSTTTAQNSS--- : 526
Barley               489 : KP-ESNAVVWSGPALLFLLFLIGIWQVYVKKKDSL-GWT-PEETFNTSVTAPTGSILNHPASERA : 550
Brachypodium         472 : RP-ESNAVVWSGPALLFLLFLIGIWQVYVKKKDSL-GWT-PEETFSTSVTAPTGSILNHPASDRA : 533
Cassava              486 : KG-EFNTMLWTSPVLFFILFLFVAWHFFAKKKEALTSWG-PDDPFSSTSTTTGVPLGSST-GERT : 547
Castor bean          496 : KG-EFNTMLWTSPVLFFILFLFGAWQFFAKKKEALTSWG-PDDPFSSPAATTGASLGSSS-GDRA : 557
Citrus               503 : KG-ESSVMSWTGPVFFFILFLFGVWHFFAKKKEALTSWG-PDDPFSSTTAATGAPIGSGA-GERP : 564
Cocoa bean           492 : KG-ESNMMLWTSPVLFFILFLFGAWQFFAKKKEALTSWG-PDDPFSSTSAANGPPLVSST-GDRS : 553
Cotton               461 : KG-ESNTMLWTSPVLFFILFLFGAWQFFAKKKEAFTSWG-TDDPFSSSSATNSAPLGSNT-GERP : 522
Cucumber             491 : KG-EFNTMVWTSPVLFFILFLFGAWHFFAKKKEALTSWG-PDDPFTATSPTTGAPMG-TGSSERA : 552
Eucalyptus           492 : KG-EFNTMLSTSPVVFFILFLFGAWYFFAKKKEALTSWG-PDDPFSSTSATTGAPLG-ATSGDRS : 553
Eutrema salsugineum  477 : KG-EFNTVLWSSPVFFFILFLFAAWHFFAKKKESLTAWG-PDDPFASTTVPSSSSSGVNSSATER : 539
Flax                 467 : KG-EFNTMLWTGPVLIFILFLFGAWQFFAKKKEALTSWG-PDDPFTTNNTSSAANSTPIGSGERS : 529
Grapevine            457 : KG-EFNTMLWTSPVLFFILFLFGAWQFFAKKKEALISWG-PDDPFVSTSAMTGAPLG-TSSGDRA : 518
Maize                452 : KP-ESNAVVWSGPALLFLLFLIGIWQVYVKKKDSL-GWT-PEETFNTSVTAPTGSLLNHPTSDRA : 513
Micromonas           659 : RPPPMNTKLWSQPAFVAAMGLIGLWQFY-RSR----G----HAAMGSSWGGPSVGKGGDGGGLDP : 714
Millet               458 : KP-ESNAVVWSGPALLFLLFLIGIWQVYVKKKDSL-GWT-PEETFNTSVTAPTGSLLNHPTSDRA : 519
Moss                 599 : KLPDFNAKLWSSPIFISVILLLVAWQLLSRKRDPAPTDN--NNAVMQIASSPASGFGYRENRDGA : 661
Peach                482 : KG-EFNTMLWTSPVFFFVLFLFGAWQFFAKKKEALTSWG-PDDPFSSTSATTGAPLGESSTGDRS : 544
Poplar               499 : KG-EFNTMLWTSPVLFFILFLFGAWQFFAKKKEALTSWG-PDDPFSSTSATTGAPLGSSASADRS : 561
Sorghum              462 : KP-ESNAVVWSGPALLFLLFLIGIWQVYVKKKDSL-GWT-PEETFNTSVTAPTGSLLNHPTSDRA : 523
Soybean              470 : KG-EFNTMLWTSPVLFFILFLFGAWHFFAKKKEALTSWG-PDDPFSSTSATTSAPLA-SGSGDRS : 531
Strawberry           463 : KG-EFSTMLWTSPVFFFVLFLFGAWHFFAKKKEALTSWGGPDDPFSSTSATAGAPLGGNSSGERS : 526
Tomato               485 : KN-EFNTMLWTSPVLFFIIFLLGAYYFFAKKKEALTSWG-PDDPFPSTGVTSGAPMG-SSQGDRS : 546
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Fig. S3. Alignment of MHZ3 protein sequences from different species reveals several conserved domains or motifs. MHZ3 homologous protein sequences were searched in Phtozome v9.1 (http://www.phytozome.net) or in NCBI (http://blast.ncbi.nlm.nih.gov) with MHZ3 as the query sequence. The sequences were aligned using ClustalX version 1.8. The numbers indicate the positions of amino acid residues. Black and gray shadings indicate identity and similarity of amino acid residues, respectively. Signal peptide and transmembrane domain are highlighted by a line above the sequences. Notably, micromonas has no transmembrane domain. Red dots indicate putative glycosylation sites (NxS/T). The mutation sites in *mhz3-4* and *mhz3-5* are indicated. Accession numbers are as follows: Apple, *Malus domestica* MDP0000283359, Arabidopsis, *Arabidopsis thaliana* AT1G75140, Barley, *Hordeum vulgare* BAJ86926, Brachypodium, *Brachypodium distachyon* Bradi1g50520, Cassava, *Manihot esculenta* cassava4.1_003423m, Castor bean, *Ricinus communis* 30170.m014014, Citrus, *Citrus clementina* Ciclev10000534m, Cocoa bean, *Theobroma cacao* Thecc1EG034012t1, Cotton, *Gossypium raimondii* Gorai. 010G011100.1, Cucumber, *Cucumis sativus* Cucsa.232290.1, Eucalyptus, *Eucalyptus grandis* Eucgr.F01908.1, Eutrema salsugineum, *Eutrema salsugineum* Thhalv10018268m, Flax, *Linum usitatissimum* Lus10007163, Grapevine, *Vitis vinifera* XP_002281580, Maize, *Zea mays* GRMZM2G095211_T01, Micromonas, *Micromonas pusilla* 63794, Millet, *Setaria italica* Si006045m, Moss, *Physcomitrella patens* Pp1s40_71V6.2, Peach, *Prunus persica* ppa002747m, Poplar, *Populus trichocarpa* Potri.002G262900.1, Sorghum, *Sorghum bicolor* Sb10g001250.1, Soybean, *Glycine max* Glyma14g08250.1, Strawberry, *Fragaria vesca* mrna15125.1-v1.0-hybrid, Tomato, *Solanum lycopersicum* Solyc04g080180.1.1.

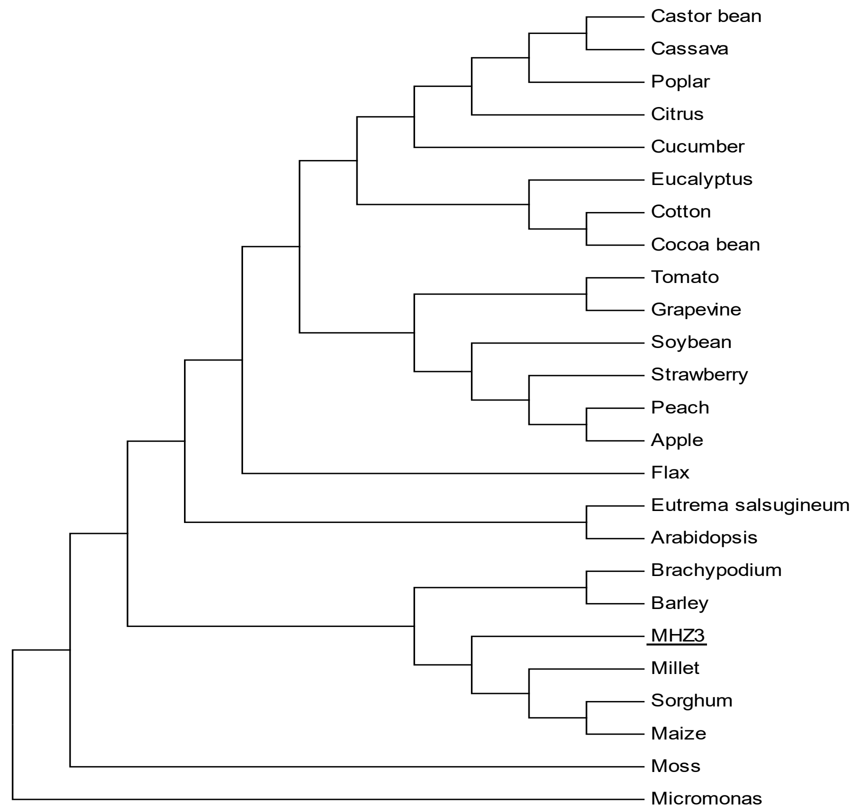

Fig. S4. Phylogenetic analysis of MHZ3 homologous proteins. A neighbor-joining tree was constructed using MEGA6.0 program (http://www.megasoftware.net) with a bootstrap setting of 1000.

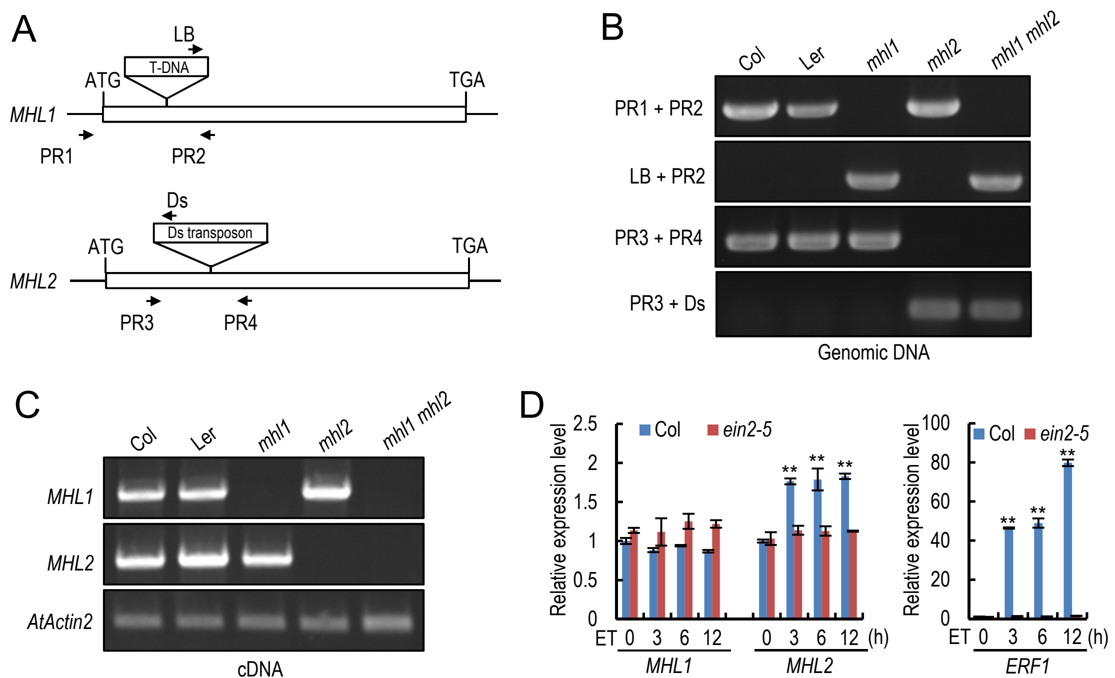

Fig. S5. Identification of *mhl1 mhl2* double mutant of Arabidopsis. (*A*) Schematic representation of gene structures of *MHL1* (*MHZ3-like1*, At1g75140) and *MHL2* (At1g19370). The T-DNA or Ds transposon insertion sites and the primer positions are indicated. (*B*) PCR genotyping for *mhl1* [SALK_118778, Columbia (Col) background] and *mhl2* [CSHL_GT12967, Landsberg (Ler) background] single mutants and *mhl1 mhl2* double mutant which is generated by crossing and back crossing. (*C*) Expression levels of *MHL1* and *MHL2* genes in the single and double mutants detected by RT-PCR analysis with amplification of the full-length cDNA. *AtActin2* was used as an internal control. (*D*) qRT-PCR analysis for the expression of *MHL1/2* in response to ethylene. *ERF1* served as a positive control. Data are means ± SD, n=3 (P<0.01; Student's *t*-test; Compared to "0h").

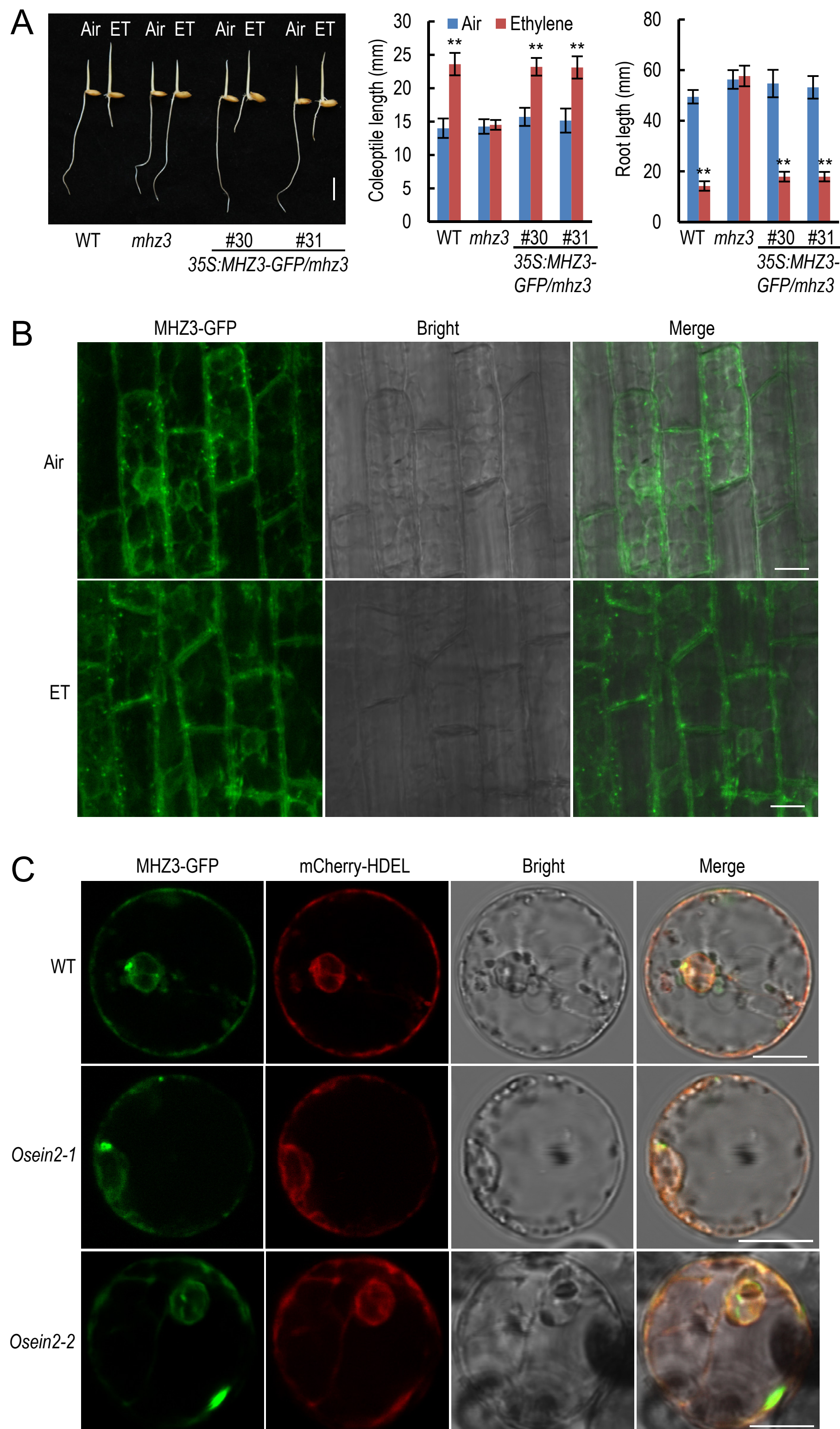

Fig. S6. The localization pattern of MHZ3 is not altered by ethylene treatment and *Osein2* mutations. (*A*) Transformation with *35:MHZ3-GFP* fusion gene rescued the ethylene-insensitive phenotype of *mhz3-1*, suggesting GFP tagging does not affect MHZ3 function. Two transgenic lines (#30 and #31) were analyzed for ethylene response. Etiolated rice seedlings were grown in the air or 10 ppm ethylene for 2.5 days. Representative seedlings are shown. Coleoptile and root lengths are means ± SD (n=20). Asterisks indicate significant difference between Air and ET (P<0.01; Student's *t*-test). Scale bar=10 mm. (*B*) The localization pattern of MHZ3 was unaffected by ethylene treatment. Dark-grown 1-d-old seedlings of *35S:MHZ3-GFP* transgenic line (#30) were treated without (Air) or with (ET) 10 ppm ethylene for 12 h. The fluorescence in coleoptile cells was detected using confocal microscopy. Scale bars=10 μm. (*C*) The localization pattern of MHZ3 was unaffected by *Osein2* mutations. *35S:MHZ3-GFP* and *35S:mCherry-HDEL* (an ER marker) constructs were cotransformed into shoot protoplasts isolated from WT, *Osein2-1* and *Osein2-2* seedlings. The fluorescence was detected using confocal microscopy. Scale bars=10 μm.

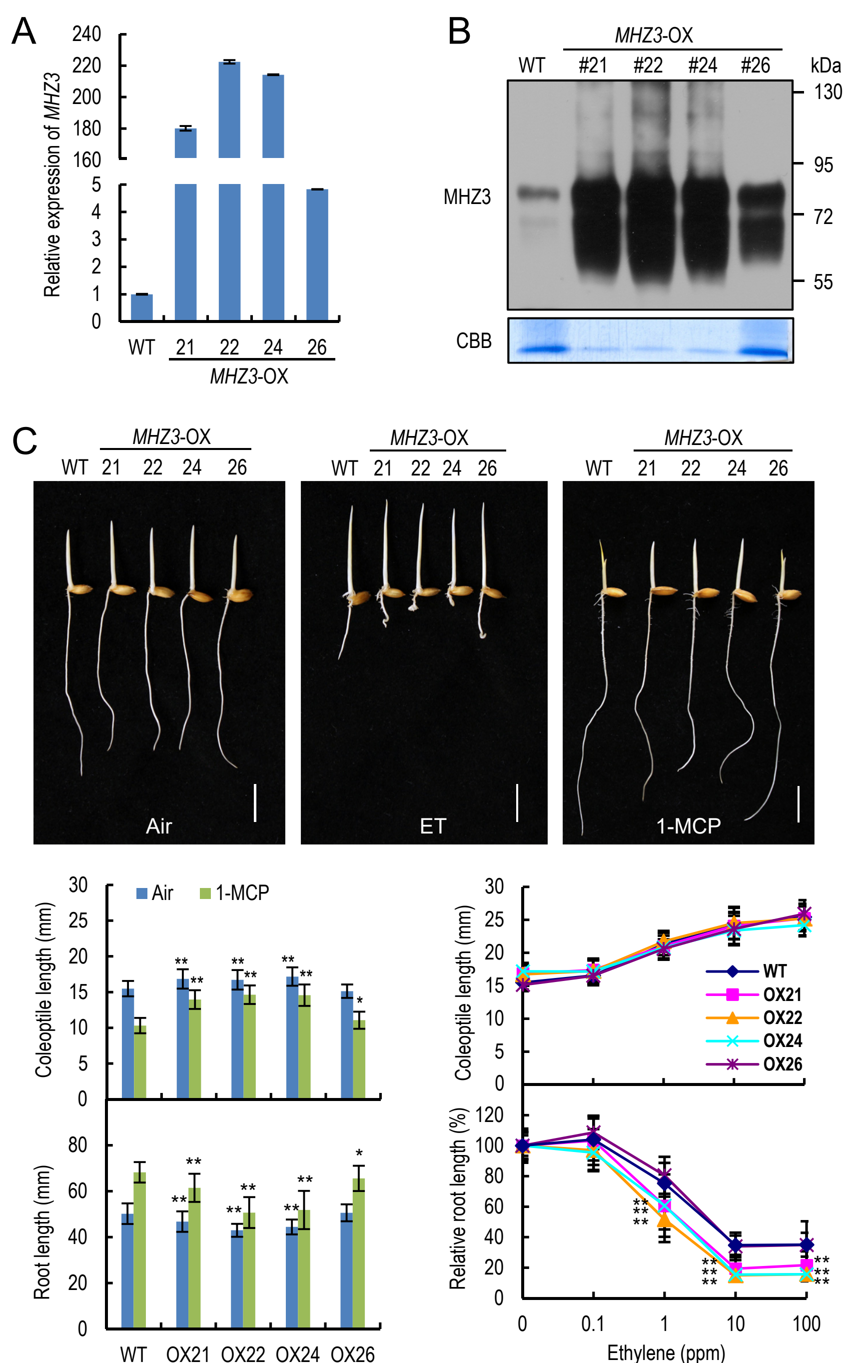

Fig. S7. *MHZ3* overexpression confers ethylene hypersensitivity. (*A*) Transcript levels of *MHZ3* in WT and *MHZ3*-overexpressing (OX) lines. Total RNAs were isolated from 3-d-old etiolated rice seedlings and subjected to qRT-PCR analysis. Data are the means ± SD of three biological replicates with two technical replicates. The expression level in WT was set to 1. (*B*) Protein levels of MHZ3 in WT and overexpressing lines. Membrane proteins were isolated from 3-d-old etiolated rice seedlings and immunoblotted with anti-MHZ3 antibody. Bottom: coomassie brilliant blue (CBB) staining of the gel was used as a loading control (30μg proteins were loaded for WT and OX26, and 5μg for OX21, OX22 and OX24). (*C*) Ethylene response phenotypes of *MHZ3*-OX lines. Etiolated seedlings of WT and *MHZ3*-OX were treated with various concentrations of ethylene or 10 ppm 1-MCP for 2.5 days. Representative seedlings are shown. Coleoptile and root lengths are means ± SD (n>25). Asterisks indicate significant difference compared to WT (*P<0.05, **P<0.01; Student's *t*-test). Scale bars=10mm.

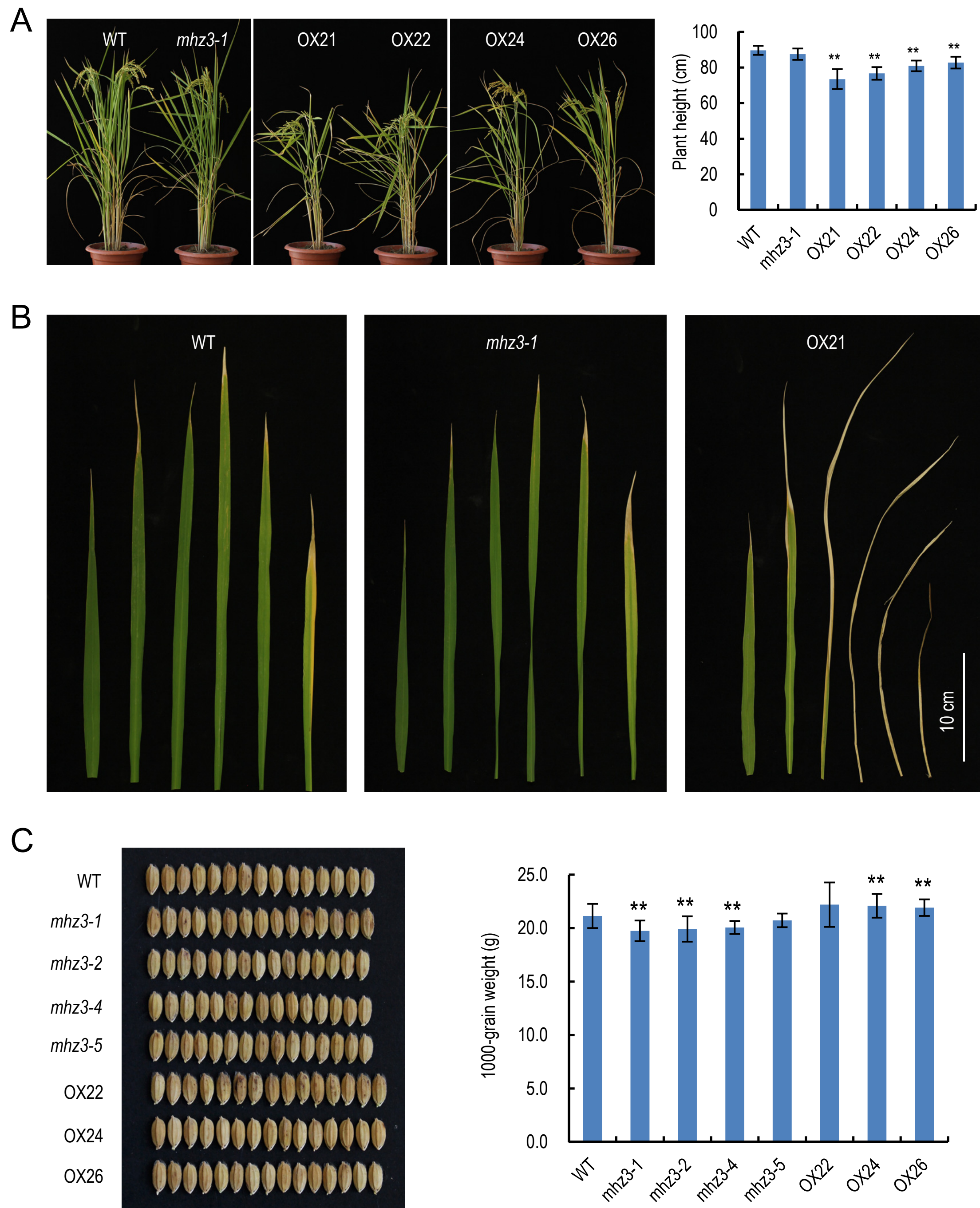

Fig. S8. Phenotypic analysis for field-grown plants of *MHZ3*-overexpressing lines. (*A*) *MHZ3* overexpression reduced rice plant height. Representative adult plants are shown. The plant heights are means ± SD (n=20). Asterisks indicate significant difference compared to WT (P < 0.01; Student's *t*-test). (*B*) *MHZ3* overexpression accelerated leaf senescence of field-grown rice plants. The leaves were cut from the same plants shown in (*A*). (*C*) Grain size and weight were increased by *MHZ3* overexpression and reduced by *mhz3* mutation. Representative grains are shown. Each value is average of 20 plants with 50 to 100 grains per plant and error bars indicate SD. Asterisks indicate significant difference compared to WT (P<0.01; Student's *t*-test).

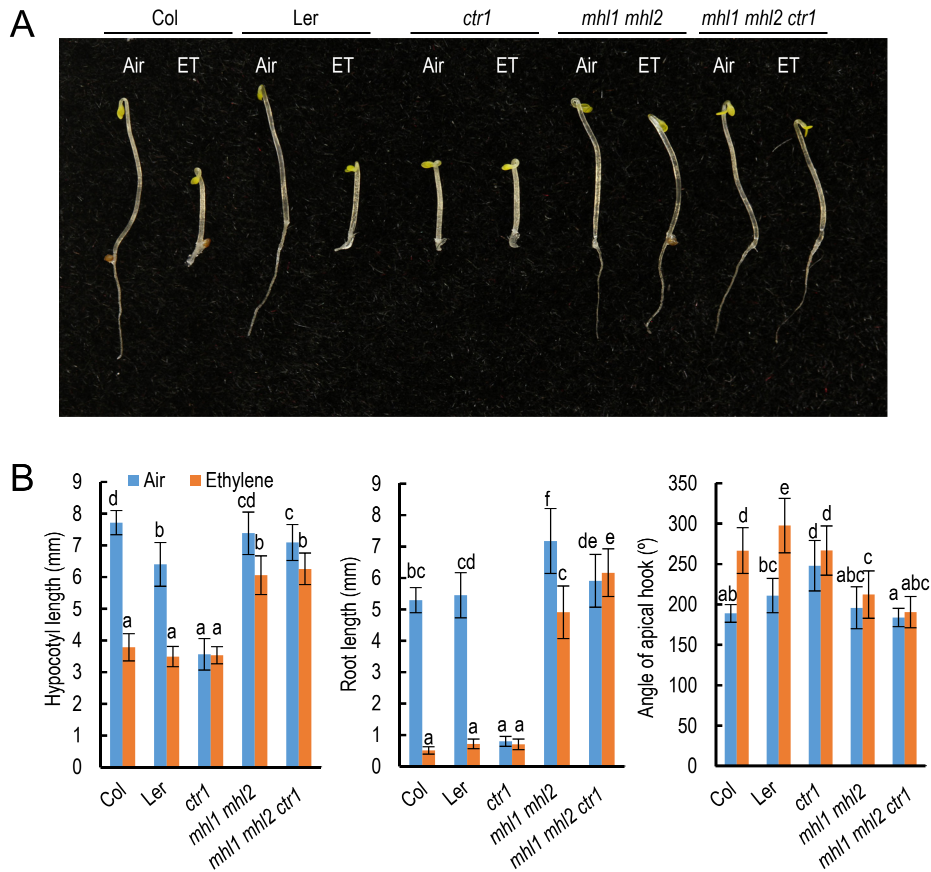

Fig. S9. Genetic interaction of *MHZ3*-like (*MHL*) genes with *CTR1* in Arabidopsis. (*A*) Ethylene response phenotype of wild types, *ctr1*, *mhl1 mhl2*, *mhl1 mhl2 ctr1*. The triple mutant *mhl1 mhl2 ctr1* was generated by crossing *mhl1 mhl2* double mutant with *ctr1-1* and backcrossed with Columbia. Etiolated seedlings were treated without (Air) or with 10 ppm ethylene for 4 days. Representative seedlings are shown. (*B*) Measurement of hypocotyl length and root length. The data are means ± SD (n=25). Different letters above each column indicate significant difference ($P < 0.01$; Student's *t*-test).

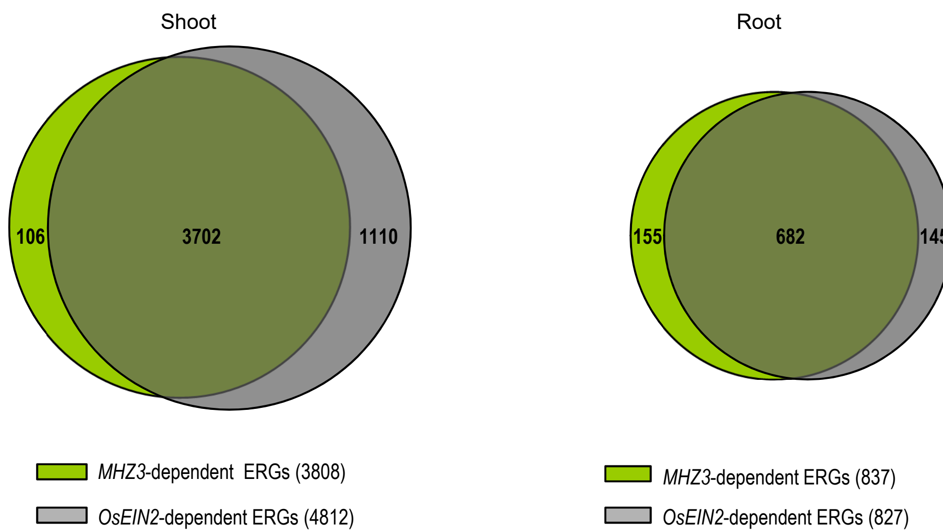

Fig. S10. Comparison of *MHZ3*- and *OsEIN2*-regulated ethylene-response genes. Two-day-old etiolated rice seedlings of WT, *mhz3-1* and *Osein2-2* were treated with air or 10 ppm ethylene for 8 h. The shoots and roots were subjected to RNA-seq analysis with two biological replicates. Ethylene-response genes (ERGs) were identified in WT according to the gene expression levels with at least relative1.5-fold changes in ethylene treatment compared to those in the air. A total of 5109 and 1045 ERGs were identified in the WT shoot and root, respectively.

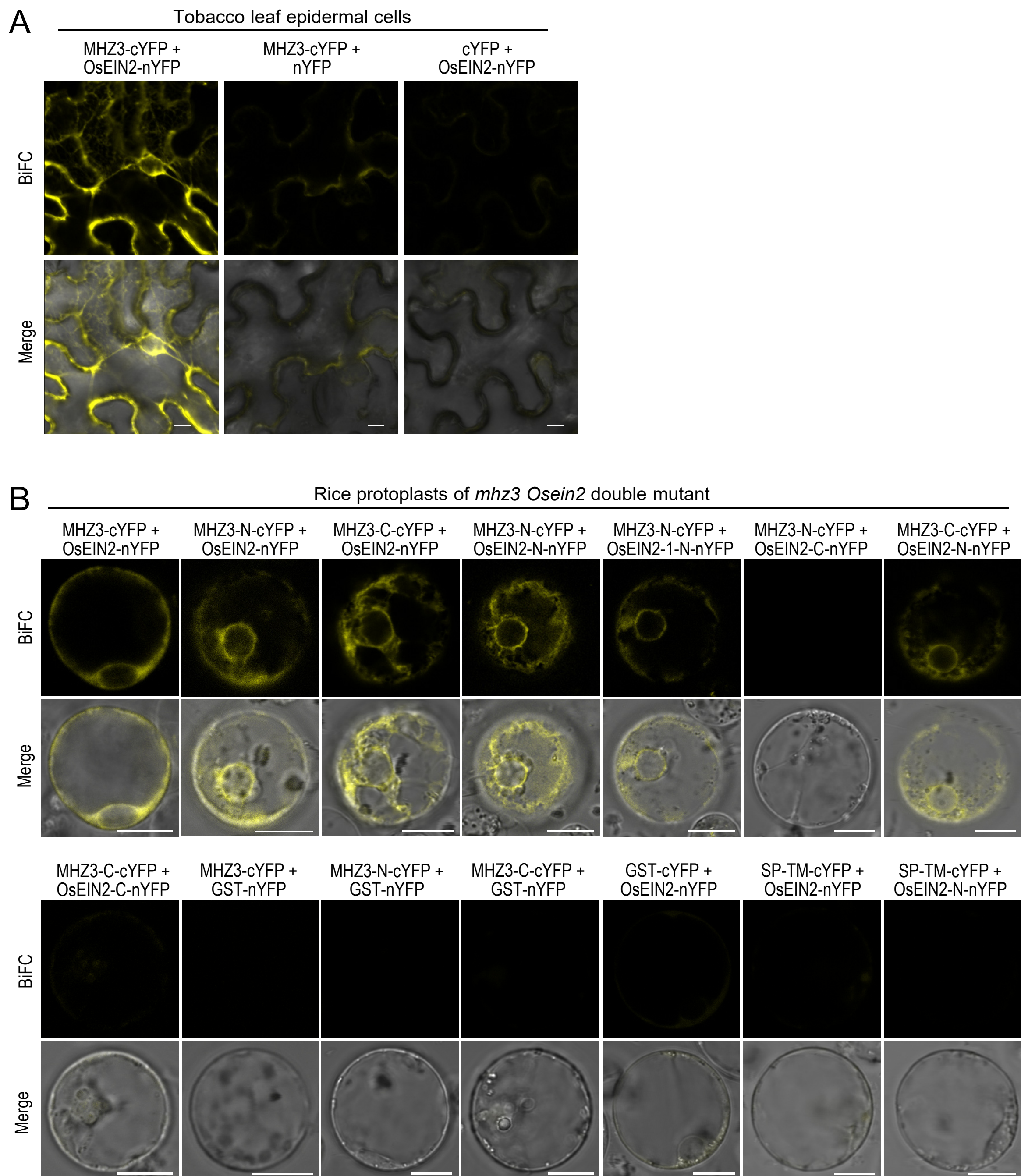

Fig. S11. BiFC assays for interaction between MHZ3 and OsEIN2. (*A*) BiFC assay in tobacco leaf epidermal cells shows interaction of MHZ3 and OsEIN2. *Agrobacterium*-infiltrated leaves were observed using confocal microscopy. MHZ3-nYFP + cYFP and nYFP + OsEIN2-cYFP were used as negative controls. Scale bars=10 μm. (*B*) BiFC assays for interaction domain-mapping of MHZ3 and OsEIN2. The full length or truncated versions of *MHZ3* and *OsEIN2* coding sequences were fused to the C-terminal half (cYFP) and N-terminal half of YFP (nYFP), respectively. The negative control SP-TM-cYFP-Myc contains the signal peptide (SP) and transmembrane domain (TM) of MHZ3. The constructs in indicated combinations were cotransformed into shoot protoplasts isolated from etiolated seedlings of *mhz3 Osein2* double mutant. YFP fluorescence were detected using confocal microscopy. Scale bars=10 μm.

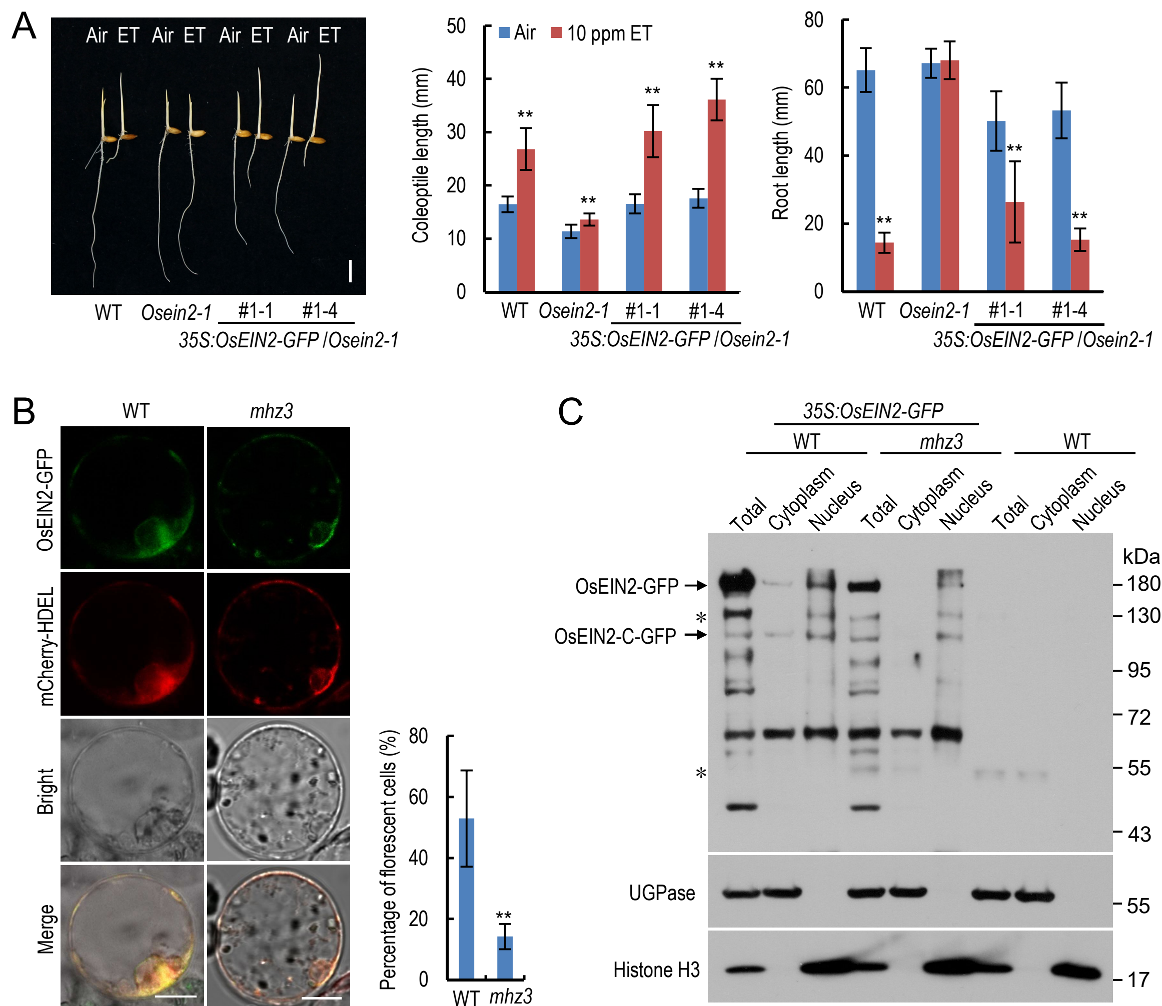

Fig. S12. MHZ3 does not affect the ER localization and nuclear translocation of OsEIN2 protein. (*A*) Transformation with *35S:OsEIN2-GFP* fusion gene rescued the ethylene-insensitive phenotype of *Osein2-1*, indicating GFP tagging does not affect OsEIN2 function. Two transgenic lines (#1-1 and #1-4) were subjected to ethylene-response assays. Etiolated rice seedlings were grown in the air or 10 ppm ethylene for 3 days. Representative seedlings are shown. Coleoptile and root lengths are means ± SD (n>30). Asterisks indicate significant difference between Air and ET (P<0.01; Student's *t*-test). Scale bar=10 mm. (*B*) The ER localization pattern of OsEIN2 was unaffected by *mhz3* mutation. *35S:OsEIN2-GFP* and *35S:mCherry-HDEL* (an ER marker) constructs were cotransformed into shoot protoplasts of WT and *mhz3* etiolated seedlings. The fluorescence was detected using confocal microscopy. The percentages of florescent cells are means ± SD, n=7 (P<0.01; Student's *t*-test). Scale bars=10 μm. (*C*) Nuclear translocation of the C-terminal OsEIN2 fragments were unaffected by *mhz3* mutation. Total proteins isolated from 3-d-old etiolated seedlings of *OsEIN2-GFP*/WT and *OsEIN2-GFP*/*mhz3* transgenic lines and WT (negative control) were fractionated into nucleus and cytoplasm fractions and immunoblotted with anti-GFP, anti-UGPase and anti-Histone H3 antibodies. Histone H3 and UGPase served as nuclear and cytosolic markers, respectively. ∗ indicates non-specific bands.

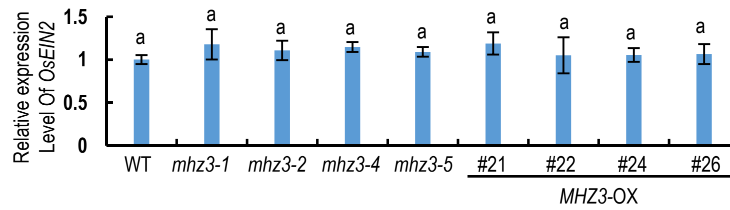

Fig. S13. Relative expression level of *OsEIN2* is unaffected by MHZ3. Total RNAs from 3-d-old etiolated seedlings were subjected to qRT-PCR analysis. The expression level in WT was set to 1. Data are means ± SD of three biological replicates with two technical replicates. The letters "a" above each column indicate no significant difference between the compared pairs (P>0.01; Student's *t*-test).

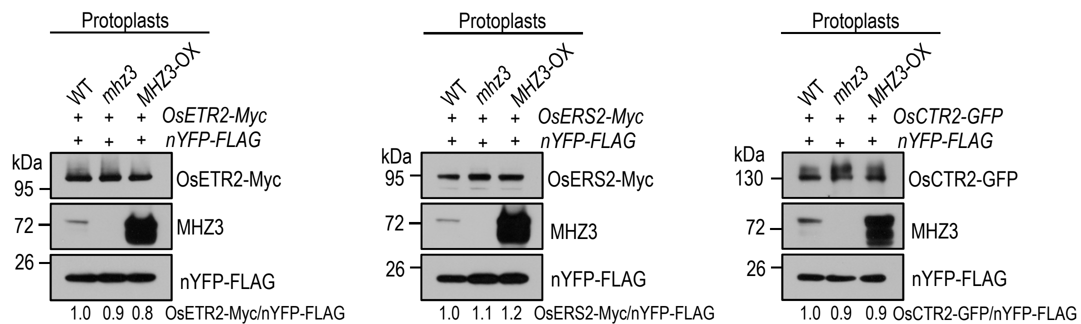

Fig. S14. MHZ3 dos not influence the protein levels of OsETR2, OsERS2 and OsCTR2. The constructs were cotransformed into shoot protoplasts isolated from etiolated seedlings of WT, *mhz3* and OX22. The nYFP-FLAG was used as control for normalizing transformation efficiency. Total proteins were immunoblotted with anti-c-Myc, anti-GFP, anti-FLAG and anti-MHZ3 antibodies. The values at the bottom indicate averages of relative protein levels of OsETR2, OsERS2 and OsCTR2 from three independent replicates.

Table S1. Primers used in this study.

Genes or constructs	Forward primer (5′-3′)	Reverse primer (5′-3′)
Map-based cloning		
Idl6-0.72	gtcctcaacagtcctgacgt	gtgaagaaacataacactatg
Idl6-1.9	gaacaccgtggtgttcttcgag	ggcttcagcttgcaagtgtct
qRT- PCR		
MHZ3	tggtgagcgaggctatgttg	taggtccacacccctggtag
OsEIN2	aactgcggagacgactgcat	aggatgccctgaagacggtt
OsERF063	acgtgatggacagcctcctc	gggaagtctgaaatggacatg
OsERF073	aatgataatcaaggcaccac	acccgaataagtgttgataac
SHR5	aataccagctatgttaccagcc	caccattacaaattacaaggagc
Germin-like	gctaattgattggctccaatc	tagcaacatatcgtgacacac
OsActin2	ttatggttgggatgggaca	agcacggcttgaatagcg
MHL1	attcggcgcttggcattttt	gctccaggaggataccgaga
MHL2	gctagcgacagggaaaacct	tggcaaagaaatgccaagcc
ERF1	aatcgagcagtccacgcaa	taggctaaagccgcctcttc
AtActin2	atgcccagaagtcttgttcc	tgctcatacggtcagcgata
CAPS or dCAPS		
mhz3-1	atgcagggtccgggcaatcaagaaagc	ccagaacccgcaaacacagact
mhz3-2	actcccgctccgctgtcact	gacggacttggcgagcgact
mhz3-4	tgtcctgcgactccggcggccggat	ggccctcgcatggtgtctcgcg
mhz3-5	tccttccgagcgcttcaaggcccatg	caaggatcttatcttggctggctacc
Identification of *mhl1 mhl2*		
mhl1	PR1: atcgtccgtcttagtcattcg	PR2: tcaaacttgccaacattttcc
	LB: tggttcacgtagtgggccatcg	
	MHL1-F: ctcctgtagatctgcataaatggc	MHL1-R: agaaaattttcaagcgttgaccgg
mhl2	PR3: actcgttaagaatctgacggaa	PR4: gcttaaatcagtcgccaatata
	Ds: taccgaccgttaccgacc	
	MHL2-F: aaatggcggttacgtactgattcag	MHL2-R: ttgaagtaagtggtgattaacgtgc
Plasmid construction		
Genetic transformation		
MHZ3 complementation	gggcaagcttcatatgaacagcaagcgaagaagac	tagtcgacgagaccggatccttcgccattgcttacctgtg
MHZ3pro-GUS	aagcttcacacgccttcctcctatcg	ggatccggttgtcagtgacagcggag
35S:MHZ3	tctagaatggcccaccacgtcgctcc	gagacctcaatcaacatgatcatcta
35S:MHZ3-GFP	ggtaccatggcccaccacgtcgctcc	tctagaatcaacatgatcatctactactgt
35S:OsEIN2-GFP	cccgggatggatgggcagcagctacg	gtcgacttgttgtcccttgctcgaga
Y2H		
MHZ3-Cub	aaggccattacggccatggcccaccacgtcgctcccctcctcct	ccggccgaggcggccccatcaacatgatcatctactactgtctggttgc
NubG-OsEIN2	aaggccattacggccatggatgggcagcagctacg	ccggccgaggcggccttgttgtcccttgctcgagag
BiFC and CoIP		
MHZ3-cYFP-Myc	gacagggtacccggggatccatggcccaccacgtcgctcc	acgctgcccatagaggatccatcaacatgatcatctacta
MHZ3-N-cYFP-Myc	gacagggtacccggggatccatggcccaccacgtcgctcc	acgctgcccatagaggatccatccttcttctttacataga
MHZ3-C-cYFP-Myc	gacagggtacccggggatccatgtcgttgggctggactcc	acgctgcccatagaggatccatcaacatgatcatctacta

Table S1. Cont.

Genes or constructs	Forward primer (5′-3′)	Reverse primer (5′-3′)
OsEIN2-nYFP-FLAG	gacagggtacccggggatccatggatgggcagcagctacg	ttgctcaccatagaggatccttgttgtcccttgctcgaga
OsEIN2-N-nYFP-FLAG	gacagggtacccggggatccatggatgggcagcagctacg	ttgctcaccatagaggatccctctttctgagaaggcacag
OsEIN2-1-N-nYFP-FLAG	gacagggtacccggggatccatggatgggcagcagctacg	ttgctcaccatagaggatccctctttctgagaaggcacag
OsEIN2-C-nYFP-FLAG	gacagggtacccggggatccatgctcttgaatactactca	ttgctcaccatagaggatccttgttgtcccttgctcgaga
SP-TM-cYFP-Myc	cgtcggaggaggcggcgccggagagcaatgctgtg	cacagcattgctctccggcgccgcctcctccgacg
	gacagggtacccggggatccatggcccaccacgtcgctcc	acgctgcccatagaggatccatagacttgccaaatgccaa
GST-cYFP	gacagggtacccggggatccatgtcccctatactaggtta	acgctgcccatagaggatccagaattcggggatcccaggg
GST-nYFP	gacagggtacccggggatccatgtcccctatactaggtta	ttgctcaccatagaggatccagaattcggggatcccaggg
OsEIN2-C-GFP	ggtacccggggatcctctatgctcttgaatactactcaagac	atgcctgcaggtcgactctttacttgtacagctcgtccatgc
OsEIN2-1-GFP	caccatggatgggcagcagctacg	ttgttgtcccttgctcgagag
OsETR2-Myc	gctctagaatgccaccgatcccatctctgt	acgcgtcgacattgttttgaaggactctatacagt
OsERS2-Myc	cgggatccatggatggatcatgtgattgca	acgcgtcgactacgcttgattggtagcgaacc
OsCTR2-GFP	ggcgcgccactagtggatccatgaaggccgacgccaag	catcccgggagcggtaccactgatatcctcttgaagttgatgg