Medicago falcata MfSTMIR, an E3 ligase of endoplasmic reticulum-associated degradation, is involved in salt stress response

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SUMMARY
Recent studies on E3 of endoplasmic reticulum (ER)-associated degradation (ERAD) in plants have revealed homologs in yeast and animals. However, it remains unknown whether the plant ERAD system contains a plant-specific E3 ligase. Here, we report that MfSTMIR, which encodes an ER-membrane-localized RING E3 ligase that is highly conserved in leguminous plants, plays essential roles in the response of ER and salt stress in Medicago. MfSTMIR expression was induced by salt and tunicamycin (Tm). msttmir loss-of-function mutants displayed impaired induction of the ER stress-responsive genes BiP1/2 and BiP3 under Tm treatment and sensitivity to salt stress. MfSTMIR promoted the degradation of a known ERAD substrate, CPY*. MfSTMIR interacted with the ERAD-associated ubiquitin-conjugating enzyme MtUBC32 and Sec61-translocon subunit MtSec61γ. MfSTMIR did not affect MtSec61γ protein stability. Our results suggest that the plant-specific E3 ligase MfSTMIR participates in the ERAD pathway by interacting with MtUBC32 and MtSec61γ to relieve ER stress during salt stress.

Keywords: endoplasmic reticulum-associated degradation, unfolded protein response, endoplasmic reticulum stress, salt stress, Medicago

INTRODUCTION
The endoplasmic reticulum (ER) controls the synthesis and folding of membrane and secreted proteins. Nearly one-third of all proteins are folded and modified in the ER. Several chaperones help proteins acquire their final form through accurate cooperation in the ER (Moreno and Orellana, 2011). Environmental or physiological factors that disturb the balance between the demand and capacity of ER protein folding induce ER stress. Cells overcome ER stress by activating the unfolded protein response (UPR), a signaling pathway that upregulates ER chaperone expression, attenuates the translation of secreted proteins, and promotes misfolded protein degradation (Malhotra and Kaufman, 2007). ERAD is a comprehensive term for the ubiquitin-proteasome pathways that degrade numerous ER proteins, including secreted proteins and integral and luminal membrane substrates (Mehnert et al., 2010; Smith et al., 2011). The ERAD pathway is conserved from yeast to mammals and plays an indispensable role in maintaining ER homeostasis, as well as in removing unfolded proteins and preventing aberrant proteins from accumulating in the ER (Hoseki et al., 2010).

ERAD occurs in the ER via the action of specific E2s, E3s, and other associated proteins. During ERAD, misfolded proteins are selectively transported from the ER into the cytosol, where they undergo ubiquitination by E3 ligases and degradation by the proteasome. E3 ligases are well known to play an important role in the ubiquitin/proteasome system (Hoseki et al., 2010).

The RING E3s of ERAD have been well studied in yeast and mammals. Yeast possess two different ERAD complexes, the Hrd1 (HMG-CoA reductase degradation 1)/Hrd3 (HMG-CoA reductase degradation 3) and Doa10 (degradation of Mat a2-10) complexes. The ubiquitin ligase Hrd1 forms a complex with Hrd3p, an ER membrane protein that has a tetraricopeptide repeat (TPR) motif (Carvalho et al., 2006). The ER luminal lectin Yos9 (yeast OS-9 homolog)
interacts with Hrd3 and Hsp70 chaperone Kar2 (a yeast homolog of BiP) and recruits proteins destined for degradation to the Hrd1p/Hrd3p complex (Denic et al., 2006; Gauss et al., 2006). Doa10 is another RING E3 present in the ER membrane in yeast (Ravid et al., 2006). The other key components of the DOA10 complex are the E2 enzymes Ubc6 (ubiquitin-conjugating enzyme 6) and Ubc7 (ubiquitin-conjugating enzyme 7). The latter is recruited to the ER membrane by Cue1 (coupling of ubiquitin conjugation to ER degradation 1) (Hirsch et al., 2009). Mammals have at least nine membrane-bound ERAD RING E3 ligases, including one Doa10 homolog (TEB4); two Hrd1 homologs (HRD1 and gp78); and other E3 ligases such as RNF5/Rma1, TRC8, Kf-1/RNF103, Nixin, RNF170, and TMEM129 (Oizmann et al., 2013; van den Boomen et al., 2014; van de Weijer et al., 2014).

Compared with the extensive literature regarding ERAD in yeast and mammals, studies in plants are limited. Recently, many homologs of yeast and mammalian ERAD E3s were discovered in plants (Liu and Li, 2014). However, a plant-specific ERAD-associated E3 has yet to be reported. The Arabidopsis genome encodes two Hrd1 homologs (AtHRD1A and AtHRD1B) (Su et al., 2011; Huttner et al., 2012), two Doa10 homologs (Doa10A/CEP9/SUD1, Doa10B/At4 g32670) (Doblas et al., 2013), and three RMA1 (RING finger protein with membrane anchor 1) homologs (Son et al., 2009, 2010). AHRD1 also interacts with an Arabidopsis homolog of yeast Hrd3 and prevents br1-9 degradation via ERAD. RMA1 homologs have been found in Medicago and Capsicum, called MKB1 and Rma1H1, respectively (Lee et al., 2009; Pollier et al., 2013). Some of these E3 ubiquitin ligases participate in abiotic stress response. CER9 is a negative regulator of cuticle lipid synthesis and ABA biosynthesis, whose deficiency increases cuticle lipid deposition and improves plant tolerance to water deficit (Lu et al., 2012; Zhao et al., 2014). Rma1H1 is involved in the degradation of the aquaporin isoform PIP2;1 to regulate its plasma membrane level. Rma1H1 overexpression in Arabidopsis suppressed PIP2;1 trafficking from the ER to the plasma membrane and enhanced drought stress tolerance (Lee et al., 2009).

As sessile organisms, plants are confronted with diverse environmental conditions. Previous studies have shown that ERAD E3 ubiquitin ligases are utilized to overcome abiotic stresses such as drought and salt. Compared with at least nine ERAD E3 ubiquitin ligases in humans, only three distinct types of E3 ubiquitin ligases of ERAD have been reported in plants. However plants face survival conditions that are no less complex conditions than those for mammals, leading to speculation that plants should contain additional, specific ERAD E3 ubiquitin ligases. However, whether plants indeed contain other ERAD E3 ubiquitin ligases and whether these E3s are involved in abiotic stress responses is unknown.

In this study, we identified a RING finger gene, MfSTMIR (Medicago falcata salt tunicamycin-induced RING finger protein, accession number MF143795), in M. falcata. Our data show that MfSTMIR is an ER membrane-associated E3 ligase that is highly conserved in leguminous plants but lacks homologs in yeast or mammals. MfSTMIR is an active ERAD component and plays a positive role in salt and tunicamycin (Tm) stress responses in plants. MfSTMIR interacts with the ER-localized ubiquitin-conjugating enzyme MtUBC32 and the protein translocator subunit MtSec61γ in vitro and in vivo. MfSTMIR may control the ubiquitination of misfolded ER proteins during exposure to abiotic stress.

RESULTS

**MfSTMIR is an ER membrane-anchored E3 ubiquitin ligase**

We previously performed abiotic stress-responsive *M. falcata* transcriptome profiling using Illumina sequencing (Miao et al., 2015). Untreated *M. falcata* PL502449 and abiotic stress-treated samples were used in RNA sequencing. We identified a contig whose expression was strongly induced by salt. The contig contains a 453-bp open reading frame (ORF) encoding a 150-amino-acids RING finger protein, which we named MfSTMIR. SMART (http://smart.embl-heidelberg.de/) analysis revealed one transmembrane domain (TMD) in the MfSTMIR N-terminal region and a RING finger domain in the C-terminal region (Figure 1a). The RING finger domain of MfSTMIR is a conserved C3H2C3-type RING finger. Neighbor-joining phylogenetic tree analysis revealed that MfSTMIR was more closely related to legume RING finger proteins that have not been reported (Figure 1b, group I), whereas MfSTMIR shares a relatively low degree of amino acid sequence identity with TMD-RING proteins from Arabidopsis. Specifically, MfSTMIR is not homologous with known ERAD-associated RING E3 ubiquitin ligases in Arabidopsis (AtHRD1A/1B, AtDoa10A/10B, AtRMA1/2/3), as well as their homologs in *Medicago truncatula* (Figure 1b, group II).

MfSTMIR was predicted to contain a TMD, implying that this protein is associated with membranes. Subcellular fractionation analysis was performed to detect MfSTMIR localization. First, we constructed an MfSTMIR-FLAG fusion cassette under the control of the cauliflower mosaic virus 35S promoter. The construct was transiently expressed in *Nicotiana benthamiana* leaf cells by *Agrobacterium*-mediated infiltration. Subsequent immunoblotting showed that MfSTMIR-3 × FLAG was present in the total and membrane fraction but not in the soluble fraction (Figure 1c). The MfSTMIR amino acid sequence was determined by PSORT prediction Protein Subcellular Localization Prediction Tool, (https://psort.hgc.jp/form.html), and the predicted localization is in the ER membrane. To determine its subcellular localization, MfSTMIR
was fused in frame to the N-terminus of green fluorescent protein (GFP), and the resulting construct (MfSTMIR–GFP) was transiently co-expressed with the well known ER marker red fluorescent protein (RFP)–HDEL in Arabidopsis mesophyll protoplasts. Confocal laser scanning microscopy of living cells revealed overlapping MfSTMIR–GFP and RFP–HDEL signals (Figure 1d), suggesting that MfSTMIR localizes to the ER.

MfSTMIR contains a single C3H2C3-type RING motif. RING motif-harboring proteins have been shown to function as E3 ubiquitin ligases (Kraft et al., 2005; Stone et al., 2005). To test whether MfSTMIR possesses ubiquitin ligase activity, in vitro ubiquitination assays were performed. MBP-MfSTMIR and MBP-MfSTMIRm (H125A and H128A) were incubated with or without E1, E2, and ubiquitin. Immunoblots were analyzed using anti-ubiquitin and anti-MBP antibody. The arrow indicates the MBP-MfSTMIR target protein.

Figure 1. MfSTMIR is an ER-membrane-localized, plant-specific E3 ubiquitin ligase. (a) Structure of full-length MfSTMIR, which contains the putative transmembrane domain (TMD) and RING finger. Asterisks indicate C3H2C3-type RING motif. (b) MfSTMIR protein is highly conserved in leguminous plants (group I) but is absent among reported TMD-RING-type E3 ubiquitin ligases (group II). The NJ method with 1000 bootstraps was applied using MEGA 5.05 software. (c) Cell fractionation assays of MfSTMIR. 35S:MfSTMIR-FLAG was infiltrated into tobacco leaves, and the samples were collected after 3 days. MfSTMIR-FLAG was detected using an anti-FLAG antibody (top panel). cFBPase is shown as a cytoplasmic protein control (middle panel), and H+-ATPase is shown as a membrane protein control (bottom panel). T, total extract; S, soluble fraction; M, membrane fraction. (d) Cellular localization of MfSTMIR–GFP. Arabidopsis protoplasts expressing MfSTMIR–GFP (left panel), RFP–HDEL as an ER marker protein (middle panel) and merged images (right panel). Scale bar: 10 µm. (e) In vitro ubiquitination assays for MfSTMIR. MBP-MfSTMIR and MBP-MfSTMIRm (H125A and H128A) was incubated with or without E1, E2, and ubiquitin.

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activity, we conducted in vitro self-ubiquitination assays. Maltose-binding protein (MBP)-tagged recombinant MfSTMIR was expressed in Escherichia coli and purified by amylose resin affinity chromatography. We constructed amino acid-substitution mutants of MBP-MfSTMIR, in which residues His125 and His128 in the RING domain were replaced with Ala (MfSTMIRm) as a negative control. Purified MBP-MfSTMIR and MBP-MfSTMIRm were incubated at 37°C for 2 h in the presence or absence of ubiquitin (Arabidopsis ubiquitin), ATP, E1 (rabbit UBE1), and E2 (human recombinant Ubch5c). Previous studies have shown that UBE1 and Ubch5c function effectively as E1 and E2 enzymes in Arabidopsis RING E3 assays (Qin et al., 2008). Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and ubiquitinated proteins were detected by immunoblotting with anti-ubiquitin antibodies, MBP-MfSTMIR produced a high-molecular-mass ubiquitinated smear when E1, E2, and ubiquitin were present, whereas no polyubiquitinated smear was observed when E1, E2, or Ub was absent or when MBP-MfSTMIR was mutated (Figure 1e). These results suggested that MfSTMIR has E3 ubiquitin ligase activity in vitro.

### MfSTMIR is upregulated by salt stress and ER stress

To examine MfSTMIR expression patterns under salt stress treatment, real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed. Total RNA was prepared from 4-week-old *M. falcata* seedlings treated with 1 m NaCl. MfSTMIR transcripts were significantly induced at 1 h and accumulated to a peak level at 12 h (approximately 1000-fold) after treatment with high-salinity stress (Figure 2a). The results proved that MfSTMIR expression was induced by abiotic stress and ER stress.

Next, we exploited the split-Luc complementation assay, which enables the detection of bioluminescence if two proteins associate when fused to the N- or C-terminal halves of firefly Luc (Chen et al., 2008). *Agrobacterium* strains carrying MfSTMIR-HA–Nluc and MtUBC32–His–Cluc constructs and/or the Cluc vector and MfSTMIRΔ129-70–HA–Nluc construct were simply mixed and infiltrated into *N. benthamiana* leaves. Leaves co-expressing different constructs were injected with D-luciferin potassium solution to examine Luc activity after infiltration for 2 days.

To verify promoter activity and confirm the stress-induced expression of MfSTMIR, the MfSTMIR promoter was used to drive the expression of the GUS reporter gene in *M. truncatula* R108. The transgenic plants were treated with stressors such as NaCl and Tm. Histochemical GUS staining in transgenic plants is showed in Figure 2c, d). The results proved that MfSTMIR expression was induced by abiotic stress and ER stress.

### MfSTMIR interacts with MtUBC32 in vivo

MfSTMIR is an ER-membrane-localized E3 that should work with E2s. E2s are a multigene family, but only a few E2s localize to the ER membrane. AtUBC32 is an ER stress-induced functional E2 that localizes to the ER membrane and is involved in ERAD (Cui et al., 2012). Therefore, we hypothesized that MfSTMIR might interact with the *Medicago* AtUBC32 homolog. We identified a putative MtUBC32 (Medtr4g121960) based on BLAST sequence analysis, which shares 62.38% identity with AtUBC32.

To test the interaction between MfSTMIR and MtUBC32, we used a split-ubiquitin yeast two-hybrid (suY2H) system, which is suitable for exploring membrane protein interactions. Before screening, we used an online tool TMHMM Server, v. 2.0 (TransMembrane prediction using Hidden Markov Models) to predict MfSTMIR and MtUBC32 topology (http://www.cbs.dtu.dk/services/TMHMM/). Both proteins are integral membrane proteins. The N-terminus of MfSTMIR is in the lumen, and its C-terminus is in the cytoplasm. The MtUBC32 topology is the same as that of AtUBC32, whose C-terminus is in the lumen and N-terminus is in the cytoplasm (Figure 3a). We constructed pBT3-STE MfSTMIR-cub as a bait vector and pPR3-N NubG-MtUBC32 as a prey vector and then co-transformed them into NMY51 to test the interaction. The results showed that MfSTMIR interacts with MtUBC32 in yeast. Furthermore, to study the interaction area of the two proteins, we constructed truncated fragments of MfSTMIR and performed a yeast two-hybrid assay. The following baits were used: MfSTMIRΔ29-70, MfSTMIRΔ71-104, and MfSTMIRΔ105-150. The results showed that amino acid sequence 29–70 of MfSTMIR was essential for the interaction with MtUBC32 (Figure 3b).

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MfSTMIR–HA–Nluc and the empty Cluc vector, as well as the MtUBC32–His–Cluc construct and MfSTMIRΔ29-70–HA–Nluc construct, did not show Luc complementation, whereas co-infiltration with Agrobacteria containing MfSTMIR–HA–Nluc and MtUBC32–His–Cluc resulted in strong Luc complementation. Immunoblotting assay shown all constructs expressed normally (Figures 3c and S7).

We further confirmed the interaction between MfSTMIR and MtUBC32 in plant cells via bimolecular fluorescence complementation (BiFC) (Waadt et al., 2008). MfSTMIR was cloned into pSPYCE (M), whose cytoplasmic C-terminus was fused with YFPC155, and MtUBC32 was cloned into pSPYNE (R), whose cytoplasmic N-terminus was fused with YFPN173. Co-transformation of these constructs into a transient expression system in Arabidopsis leaf mesophyll protoplasts allows the reconstitution of yellow fluorescent protein (YFP) if the two proteins interact, thereby producing an YFP signal. YFP fluorescence was observed in the cytoplasm with MfSTMIR–YFP_{C} and YFP_{N}–MtUBC32. Pairwise expression of MfSTMIR–YFP_{C} and YFP_{N} or MfSTMIRΔ29-70–YFP_{C} and YFP_{N}–MtUBC32 was used as the negative control (Figure 3d). These results revealed that MfSTMIR physically interacts with MtUBC32. Together with the Y2H, we concluded that the cytoplasmic part of MfSTMIR interacts with MtUBC32.

MfSTMIR is an active component of plant ERAD

Some ER-localized E3 ligases known to participate in ERAD, such as Hrd1, Doa10, and RMA1 in yeast, mammals, and plants (Delaunay et al., 2008; Hirsch et al., 2009; Chen et al., 2016b; Ruggiano et al., 2016). In the present study, MfSTMIR was identified as a stress-induced functional E3 that localizes to the ER membrane and interacts with MtUBC32. Therefore, we hypothesized that MfSTMIR is involved in plant ERAD to promote substrate degradation. To detect the function of MfSTMIR, a vacuolar carboxypeptidase (AT3G10410) mutant, CPY*, was selected as a model ERAD substrate because it was reported that AtCPY* is degraded via ERAD in Arabidopsis (Yamamoto et al., 2010).

We generated an AtCPY* construct with a C-terminal GFP, which we then co-expressed with MfSTMIR-HA in
tobacco leaves by agroinfiltration-mediated transient expression with or without the 26S proteasome inhibitor MG132, followed by immunoblotting of infiltrated leaves with an anti-GFP antibody. The immunoblotting analysis revealed that AtCPY*–GFP degradation was promoted by MfSTMIR-HA but was inhibited by MG132. By contrast, AtCPY*–GFP degradation was not accelerated by MfSTMIRm-HA (H125A and H128A), which lacked ubiquitin ligase activity (Figure 4a). Furthermore, we performed a time-course analysis of AtCPY*–GFP degradation. The result also showed that AtCPY*–GFP degradation was promoted by MfSTMIR (Figure S6). We then found two AtCPY homologs from Medicago falcate and Medicago truncatula genomic (MfCPY and MtCPY) according multiple sequence alignment, respectively. According to the report construction of AtCPY*, we introduced the G-to-R mutation in MfCPY (G231-R) and MtCPY (G230-R) to generate the MfCPY* and MtCPY*, respectively. We performed degradation assay again and used wild-type MfCPY and MtCPY as control. The results showed that MfSTMIR can promote
both MfCPY* and MtCPY* degradation, but had no effect on wild-type MtCPY and MfCPY (Figure 5). Collectively, our results indicated that MfSTMIR participates in ERAD in plants.

Mutants of some plant ERAD components have been reported to affect the UPR (Huttner and Strasser, 2012). To investigate the role of MfSTMIR in the ER stress response, transgenic *M. truncatula* and mutant plants were generated. Because generating *M. falcata* tissue cultures is difficult, *M. truncatula* R108 was used for routine transformation. We generated 35S: MfSTMIR transgenic lines and MtSTMIR RNAi plants for further study. Positive transgenic plants were identified by PCR, and expression was detected by qRT-PCR (Figures 4b, c, S2 and S3). No *M. falcata* mutant stocks are available, but mutant seed stocks of its close relative *M. truncatula* can be found. The

**Figure 4.** MfSTMIR is an active ERAD component. (a) MfSTMIR facilitates the degradation of AtCPY* in tobacco leaves. Either MfSTMIR-HA or MfSTMIRm-HA was co-expressed with AtCPY*-GFP by agroinfiltration in *N. benthamiana*. Tissues were harvested 3 days after infiltration. MG132 (50 μM), an inhibitor of the 26S proteasome, was infiltrated 12 h before sampling, with DMSO (–) serving as a control. HA-GFP was expressed as an internal control. (b, c) qRT-PCR analysis of MfSTMIR and MtSTMIR in transgenic *Medicago* OE and RNAi plants. (d, e) qRT-PCR analysis of MtBiP1/2 and MtBiP3 transcripts in OE, RNAi mtstmir mutant and R108 plants after 2 days treatment with 10 μg ml⁻¹ Tm or equivalent volume of DMSO. Means on bars with different letters (a–d) are significantly different (*P* < 0.05) by Duncan’s multiple range test. (f) The phenotype of MfSTMIR-overexpressing transgenic *Arabidopsis* after Tm treatment. Seeds were germinated and grown on MS medium containing Tm or DMSO. After 8 days, the phenotypes were photographed. (g) Statistics of the percentage of green cotyledons in different plant material. The asterisk indicates a significant difference between MfSTMIR-overexpressing transgenic *Arabidopsis* and wild-type Col-0 plants (Kruskal-Wallis non-parametric test, **P** < 0.01).
M. truncatula genome encodes one MfSTMIR homolog (MtSTMIR/Medtr3g086630), and MfSTMIR shares 91.67% amino acid sequence identity with MtSTMIR. We screened the Tnt1 retrotransposon-mutagenized M. truncatula R108 population and isolated one MtSTMIR Tnt1 insertion line, NF7550, which was named mtstmir-1. Tnt1 insertions were located in the exon of mtstmir. The expression of full-length MtSTMIR mRNA was disrupted (Figure S4). No morphological differences between mutant and wild-type plants were detected throughout the entire developmental period under normal growth conditions, and this line was used for further analysis.

To test the function of MfSTMIR in the ER stress response, 3-day-old seedlings overexpressing MfSTMIR or RNAi, as well as mtstmir-1 and wild-type, were treated with 10 \(\mu\)g ml\(^{-1}\) Tm for 2 days. Bip1/2 and Bip3 are chaperones that accumulate in plants in response to stressors that trigger the UPR (Xu et al., 2013; Carvalho et al., 2014; Fernandez-Bautista et al., 2017). Therefore, we use these genes as UPR-activation indicators. Medtr8g099795 and Medtr8g099945 are the Medicago homologs of AtBiP1/2 and AtBiP3, respectively. MtBiP1/2 and MtBiP3 expression levels were analyzed by qRT-PCR, which showed that their expression was significant lower in the mtstmir-1 and RNAi lines and markedly higher in the MfSTMIR-overexpression lines than in the wild-type under Tm treatment (Figure 4d, e). But there was no significant difference between MtSTMIR RNAi knockout lines and MtSTMIR knockout line, so we detected the expression of MtSTMIR in these plants. Though the RNA interference efficiency was only 20–30% in MtSTMIR knockout plants under control condition, after Tm treatment, the induction expression of MtSTMIR was disturbed in MtSTMIR knockdown lines, which result in the similar transcript levels of marker genes (Figure 4c).

Because MfSTMIR promoted ERAD substrate degradation and affected ER stress-induced marker gene expression, we hypothesized that mtstmir mutants and 35S-MfSTMIR plants would have an altered response to ER stress. To test this hypothesis, MfSTMIR-overexpressing transgenic Arabidopsis was constructed, and three independent lines confirmed at the DNA and RNA level, the MfSTMIR overexpression transgenic Arabidopsis line2, line3, and line6 were used in subsequent experiments.
after growth for 8 days, and the \textit{MfSTMIR}-overexpression transgenic Arabidopsis lines had a higher green cotyledon proportion than the wild-type ($P < 0.01$) (Figure 4f(iii), g). No major differences were observed between seedlings grown on control plates containing an equivalent volume of dimethyl sulfoxide (DMSO) (Figure 4f(i)). These data indicated that \textit{MfSTMIR} overexpression enhanced the tolerance of transgenic Arabidopsis to Tm. Therefore, \textit{MfSTMIR} is an active ERAD component.

\textbf{MfSTMIR is a positive factor in the salt stress response}

Mutants with UPR or ERAD defects show increased sensitivity to various environmental stresses (Huttner and Strasser, 2012). Previous work demonstrated that salt stress responses in Arabidopsis utilize a signal transduction pathway related to ER stress signaling, triggering ERAD (Liu et al., 2011; Liu and Howell, 2016). Because \textit{MfSTMIR} is an active ERAD component, we hypothesized that \textit{mtstmir} mutant plants would display altered responses to salt stress.

After the R108, \textit{MfSTMIR}-overexpression, \textit{MfSTMIR}-Tnt1 mutant and RNAi lines were germinated for 24 h, the seedlings were transferred to Fahraeus medium plates without (mock) or with 100 mM NaCl and the grown for 3 weeks to observe their phenotypes. Seedlings from the mutants and RNAi lines were weaker than the wild-type and overexpression lines under salt stress (Figure 6a). Etiolation was observed in approximately 23.5% of R108 seedlings, whereas more than 45% of mutant and RNAi seedlings were etiolated. The percentage of etiolated seedlings in the \textit{MfSTMIR}-overexpression lines was lower than that for R108 and was 19.4 and 17.7% for OE17 and OE19, respectively (Figure 6b). Root growth of mutants and RNAi lines were more seriously restrained than wild-type, while overexpression \textit{MfSTMIR} alleviated the root growth inhibition under salt stress condition (Figure 6c, d). These data showed that the \textit{mtstmir} mutants and RNAi lines were more sensitive to NaCl than wild-type plants, whereas \textit{MfSTMIR}-overexpression lines were somewhat tolerant. This result suggested that \textit{MfSTMIR} is involved in the salt response. One likely reason is that ERAD is partially defective in mutant and RNAi plants.

Additionally, we verified \textit{MfSTMIR} function in Arabidopsis. The \textit{MfSTMIR}-overexpression transgenic Arabidopsis line2, line3, and line6 were subjected to salt stress treatment to confirm the role of \textit{MfSTMIR} in salt stress. Five-day-old transgenic and wild-type seedlings were transferred to MS medium containing 150 mM NaCl and normal MS medium. After growth for 8 days, the root lengths of \textit{MfSTMIR}-overexpressing lines were significantly longer than those of the wild-type under salt treatment ($P < 0.01$), whereas normally growing plants showed no significant difference (Figure 6e, f). Therefore, \textit{MfSTMIR} is a positive factor in the salt stress response in plants.

\textbf{The interaction between \textit{MfSTMIR} and MtSec61\gamma does not affect the stability of MtSec61\gamma}

To investigate the molecular mechanism of \textit{MfSTMIR} in response to ER or salt stress, we attempted to identify its potential interacting proteins. We screened the \textit{M. truncatula} cDNA library using the suY2H system. We obtained several initial positives clones that we isolated and retested (Table S1). One clone, MtSec61\gamma (Medtr5g084060), which is a subunit of the Sec61 complex, was selected as a candidate interaction protein.

BLAST sequence analysis in \textit{Medicago} identified two putative MtSec61\gamma isoforms (MtSec61\gamma, Medtr5g084060; MtSec61\gamma-B, Medtr4g127150), but both genes produced the same protein. We therefore chose MtSec61\gamma for subsequent experiments. We cloned the full-length MtSec61\gamma CDS and examined whether the protein interacted with \textit{MfSTMIR} using suY2H analysis. pBT3-STE \textit{MfSTMIR}-Cub, \textit{MfSTMIR}D29-70-Cub, \textit{MfSTMIR}A71-104-Cub, and \textit{MtSec61}\gamma105-150-Cub were used as bait. pPR3-N NubG-MtSec61\gamma was the prey construct. Using the yeast split-ubiquitin assay, we found that MtSec61\gamma interacted with \textit{MfSTMIR} and that the cytoplasmic region 29-70 of \textit{MfSTMIR} was essential for the interaction with MtSec61\gamma (Figure 7a). This result also showed that the N-terminus of MtSec61\gamma is in the cytoplasm. The topology of other eukaryotic Sec61\gamma, which come from canine and yeast, shows the same orientation (Esnault et al., 1994; Beswick et al., 1996).

We next confirmed the interaction between MtSec61\gamma and \textit{MfSTMIR} by firefly Luc complementation imaging in \textit{N. benthamiana}. MtSec61\gamma was fused with Cluc, and the MtSec61\gamma–His–Cluc construct was transformed into \textit{Agrobacterium}. The \textit{Agrobacterium} strains carrying MtSec61\gamma–His–Cluc and \textit{MfSTMIR}–HA–Nluc or \textit{MfSTMIR}D29-70–HA–Nluc were then infiltrated into \textit{N. benthamiana} leaves. A strong luminescence signal was observed between MtSec61\gamma–His–Cluc and \textit{MfSTMIR}–HA–Nluc, whereas no signals were detected from MtSec61\gamma–His–Cluc and \textit{MfSTMIR}D29-70–HA–Nluc or \textit{MfSTMIR}–HA–Nluc and Cluc. Immunoblotting shown all the constructs expressed normally (Figures 7b and S7).

Furthermore, we performed BiFC to study their interaction in plant cells. Full-length MtSec61\gamma cDNA was fused to YFP\textsubscript{N}I\textsubscript{155}, \textit{MfSTMIR} was fused to YFP\textsubscript{C}\textsubscript{156-328}, and the two constructs were co-transformed into Arabidopsis protoplasts. As a control, the empty vector and pSPYCE(M)–\textit{MfSTMIR}D29-70 in combination with each fusion construct were also co-transformed into protoplasts. The protoplasts
were then incubated overnight, and YFP signals were observed via fluorescence microscopy. Samples co-transformed by pSPYNE(R)-MtSec61c and pSPYCE(M)-MfSTMIR yielded YFP fluorescence, whereas all of the samples co-transformed with the negative controls and either pSPYNE(R)-MtSec61c or pSPYCE(M)-MfSTMIR failed to yield any...
Figure 7. MfSTMIR interacts with MtSec61\textgamma but does not affect its protein stability. (a) Interaction of MfSTMIR with MtSec61\textgamma via yeast two-hybrid assay Cub-LexA-VP16 fused to MfSTMIR, MfSTMIR\textsubscript{D29-70}, MfSTMIR\textsubscript{D71-104} and MfSTMIR\textsubscript{D105-150}, and NubG fused to MtSec61\textgamma were co-expressed in yeast cells carrying the reporter genes ADE2 and HIS3. The yeast cells were grown on the appropriate medium (which did not contain the indicated amino acids but contained 30 mM 3-AT). pPR3-N and pAI-Alg5 with pBT3-STE MfSTMIR-Cub were used as negative and positive controls. (b) Firefly luciferase complementation assay for interactions between MfSTMIR and MtSec61\textgamma in N. benthamiana leaves. The leaves of N. benthamiana were infiltrated with Agrobacterium strains containing the indicated plasmid pairs: MfSTMIR (MfSTMIR–HA–Nluc), MfSTMIR\textsubscript{D29-70} (MfSTMIR\textsubscript{D29-70}–HA–Nluc) and MtSec61\textgamma (MtSec61\textgamma–His–Cluc). Luminescence was detected in infiltrated leaves using a cooled CCD imaging apparatus. Immunoblotting were analyzed using anti-HA and anti-His antibody. (c) BiFC assay for interactions between MfSTMIR and MtSec61\textgamma in Arabidopsis protoplasts. Plasmids expressing the indicated split YFP variants were introduced by PEG. Scale bar: 20 \textmu m. (d) Detecting the effect of MfSTMIR on MtSec61\textgamma degradation. MtSec61\textgamma degradation was assessed by mixing cell extracts from separately infiltrated MfSTMIR-3\textsuperscript{9}HA and 6\textsuperscript{9}Myc-MtSec61\textgamma samples. ATP or MG132 was added to the mixture to a final concentration of 10 \textmu M or 50 \textmu M to promote or prevent protein degradation via the 26S proteasome. The reaction was carried out at 4°C. Samples were collected at different time points and then transferred to loading buffer to stop the reaction, followed by analysis with an anti-Myc antibody to detect Myc-MtSec61\textgamma. Ponceau S staining of the Rubisco protein is shown as a loading control. (e) Detection of the effect of GFP on MtSec61\textgamma degradation as a negative control. All procedures were performed as in (d).
YFP signal (Figure 7c). These results indicated that MfSTMIR interacts with MtSec61γ in Arabidopsis protoplasts.

Given that MfSTMIR is an E3 ubiquitin ligase, its interactors may be substrates or cooperators. To address this question, we infiltrated Agrobacterium host constructs expressing MfSTMIR-HA and Myc-MtSec61γ into the same leaf area of *N. benthamiana*, using HA-GFP as a negative control. Samples were then collected for protein extraction using a native extraction buffer, after which the samples were mixed to perform the degradation assay. ATP was added to the cell lysates to preserve the function of the 26S proteasome, and MG132 was added to cell lysates to inhibit the 26S proteasome. Aliquots were collected at different time points, mixed with loading buffer to stop the reaction and analyzed using an anti-Myc antibody to determine the status of the MtSec61γ protein. Intact Myc-MtSec61γ was stable from 0 to 5 h in the presence of MfSTMIR-HA or HA-GFP, and neither ATP nor MG132 had any effect on MtSec61γ protein stability (Figure 7d, e). These results imply that MtSec61γ stably interacts with MfSTMIR as a cooperator.

**DISCUSSION**

To date, despite the rapid progress in our understanding of ERAD in plants, whether plant ERAD involves plant-specific components remains unknown, as previous studies have identified homologs of known factors only from yeast/mammalian ERAD pathways. In the present study, we showed that MfSTMIR is a plant-specific E3 ligase involved in ERAD in *Medicago*. First, MfSTMIR is an ER membrane-localized E3 ligase. Second, MfSTMIR expression was induced by salt and Tm, which are known ER inducers. Third, MfSTMIR functions as an ER-associated E3 ligase. Fourth, MfSTMIR is a positive factor in salt stress. Finally, MfSTMIR interacted with MtUBC32 and Sec61γ.

RING finger proteins containing a transmembrane have been reported in many studies (Ko *et al.*, 2006; Bu *et al.*, 2009; Sato *et al.*, 2009; Li *et al.*, 2011). However, few ER membrane-localized RING finger proteins have been reported (Lee *et al.*, 2009; Su *et al.*, 2011; Doblas *et al.*, 2013). Our data showed that MfSTMIR localizes to the ER membrane. BLAST searches of protein databases failed to identify MfSTMIR homologs in fungi or animals but revealed highly similar proteins in leguminous plants. Furthermore, MfSTMIR is not a homolog of any known ER-membrane-localized E3 ligase (Figure 1b).

Many abiotic stresses, including salt and drought stress, disrupt protein folding and assembly, resulting in cellular damage. Cells activate the transcription response to overcome stress (Zhang *et al.*, 2016; Wang *et al.*, 2017). A unique pathway called UPR is activated to protect the ER from being damaged by misfolded or unfolded proteins (Liu *et al.*, 2007, 2008). ER stress signals activate ER-membrane-associated bZIP transcription factors, such as bZIP17, bZIP28, and bZIP60 in Arabidopsis. bZIP17 and bZIP28 transduce stress signals from the ER to the nucleus during UPR by translocating to the Golgi apparatus, where they are processed by Golgi-resident proteases. Additionally, the nuclear translocation of Arabidopsis bZIP60 has been found to be regulated by the nonconventional splicing of Arabidopsis bZIP60 mRNA by IRE-1 (Deng *et al.*, 2011). The processed forms of bZIPs are imported into the nucleus to activate stress response genes, such as chaperones and ERAD components (Liu and Howell, 2010). UPR genes are induced through the recognition of cis-acting elements in their promoter regions by UPR transcription factors. We found that the MfSTMIR promoter region contains the cis-element UPRMOTIFII (CC-N12-CCACG) (Figure S1). MfSTMIR expression was induced by salt and Tm (Figure 2a, b). These data indicated that MfSTMIR is a UPR gene and may be regulated by ER stress-activated bZIP transcription factors. Therefore, MfSTMIR expression in bZIP mutants may be worth studying.

Knowledge of ERAD has mainly been obtained from studies of yeast and mammals, but little is known about plant ERAD components and their effects on plant development, growth and stress responses. In Arabidopsis, *hrd1a* and *hrd3a* are sensitive to NaCl (Huttner and Strasser, 2012). In our study, we demonstrated that the *M. falcata* ubiquitin ligase MfSTMIR, *mtstmir* and RNAi plants were also sensitive to salt. A reasonable explanation is that the ERAD pathway is partially defective in mutant and RNAi plants, enabling increased accumulation of unfolded proteins. Other reports have shown that ERAD-component mutants are more tolerant to stress. For example, *ubc32* is more tolerant to NaCl and Tm, and *cer9* is more resistant to water deficits (Cui *et al.*, 2012; Lu *et al.*, 2012). This discrepancy might be explained by different ERAD substrates being degraded by different E3 ligase complexes. In the present study, the known ERAD substrate CPY* was used to demonstrate that MfSTMIR is involved in ERAD, but this substrate is not directly related to stress responses in *Medicago*. Exploring direct stress-related substrates in *Medicago* would help better explain the function of MfSTMIR.

Previous studies have suggested that ER membrane-associated ligases should work with other ERAD components. Because ubiquitination components such as ubiquitin and ubiquitin-conjugating enzymes are absent from the ER lumen, all ERAD substrates in the ER lumen must be retro-translocated to the cytosol for degradation (Menhert *et al.*, 2010). In the present study, we identified two MfSTMIR-interacting proteins that are considered ERAD cofactors, one of which, MtUBC32, is a homolog of AtUBC32 and an ER membrane-localized E2. We confirmed that MfSTMIR and MtUBC32 interact, and we should be able to detect the enzyme activity of MtUBC32.
The other MfSTMIR-interacting protein that we identified was MtSec61γ, and studies on Sec61γ in plants are rare. One study suggested that Sec61γ plays a negative role in barley innate immunity to *Blumeria graminis* (Bgh) (Xu et al., 2015). In yeast, Ss1p/Sec61γ, Sec61p/Sec61x and Sbh1p/Sec61β compose a protein-conducting channel for secretory and transmembrane proteins. Ss1p can help maintain the membrane permeability barrier by acting as a place-holder for signal peptides within the Sec61 complex (Falcone et al., 2011). Genetic interaction studies also highlight the involvement of Sec61p in ERAD (Plemper et al., 1999). Protein interaction studies indicate an interaction between Sec61p and Hrd3p (Schafer and Wolf, 2009). In our studies, MfSTMIR interacted with Sec61γ and did not affect its protein stability. MfSTMIR also interacted with MtUBC32, an ER-associated E2. Moreover, MfSTMIR promoted the degradation of CPY*, a homolog of yeast CPY. CPY was degraded in association with Sec61p in yeast. Taken together, we postulated that MfSTMIR interacts with the Sec61 complex via Sec61γ and uses MtUBC32 as an E2 for ubiquitination. Further experiments are needed to test this model.

Recent studies have shown that two ERAD ubiquitin ligase complexes can negatively regulate each other under normal conditions, with this negative regulation being lost under ER stress. Arabidopsis UBC32, an ER-bound E2 that affects its protein stability. MfSTMIR also interacted with MtUBC32, an ER-bound E2 that partners with DOA10, is essential for the degradation of AtOS9, a component of the HRD1 complex (Chen et al., 2016a). UBC32 degradation is mediated by the HRD1 complex, the other E3 complex involved in ERAD (Chen et al., 2016b). MfSTMIR is a specific ERAD component, and whether it is regulated by these two complexes remains an open question. Research in this field will help us better understand the function of MfSTMIR.

In conclusion, our study demonstrated that MfSTMIR is a specific ERAD E3 ligase. MfSTMIR is a positive factor in salt stress responses. MfSTMIR may participate in ERAD through its interaction with MtUBC32 and MtSec61γ to relieve the ER burden in salt stress.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

*Meditago falcata* (accession number PI502449) seeds were identified as diploid and provided by United States Department of Agriculture (USDA). The seeds of *M. truncatula* cv. R108 were provided by BRC, UMR 1097, INRA, Montpellier, France. *M. truncatula* Tnt1 MfSTMIR mutants were obtained from the Noble Foundation and were genetically characterized (Tadege et al., 2008). The seeds were sterilized and grown as described previously (de Lorenzo et al., 2009). The full-length 453-bp MfSTMIR cDNA was cloned into the pMD32 overexpression vector through BamHI and EcoRV sites and the RNAi vector pANDA35HK through KpnI and SpeI sites. Then, *A. tumefaciens* strain EHA105 carrying the 35S:MfSTMIR construct or RNAi construct was transformed into *M. truncatula* cv. R108 as described previously (Cosson et al., 2015). For normal growth conditions, plants were grown in a soil/vermiculite (1:3, v/v) mixture at 22°C under a 14-h light/10-h dark cycle, with 70% relative humidity.

The 35S:MfSTMIR vector was transformed into Arabidopsis wild-type Col plants using the floral dip method (Clough and Bent, 1998). For normal growth conditions, plants were grown in a soil/vermiculite (1:3, v/v) mixture at 22°C under a 16-h light/8-h dark cycle, with 70% relative humidity.

The primers used in experiment were showed in Table S2.

**Salt stress treatment**

Salt stress treatments of transgenic plants overexpressing MfSTMIR, RNAi and *M. truncatula* Tnt1 mstmir seedlings were performed as described (de Lorenzo et al., 2007). Briefly, sterilized seeds were plated on 0.8% agarose medium; grown for 3 days at 4°C, followed by growth in the dark for 1 day at 24°C, and then transferred to Fahraeus medium without (mock) or with 150 mM NaCl (salt stress) for 3 weeks. The phenotype was photographed, and the etiolation rate in plants was investigated. The values are presented as the mean ± standard deviation. Tests for significance were conducted using Duncan’s multiple range test with SPSS statistical software.

For transgenic Arabidopsis plants overexpressing MfSTMIR, salt stress treatments were performed as described (Shi et al., 2003). After germination on MS medium for 5 days, seedlings were subsequently grown on MS medium without (mock) or with 150 mM NaCl for 8 days. The phenotype was photographed, and the root length was determined. Tests for significance were conducted using the non-parametric Kruskal–Wallis test using SPSS statistical software.

**Tm treatment**

In the plate system, Tm (CAS No:11089-65-9, Sigma (Sigma-Aldrich Chemie GmbH, Munich, Germany); dissolved in DMSO) was directly added to Fahraeus medium containing 0.4% phytagel. Seeds were germinated directly in Tm-containing medium to observe ER stress tolerance. To harvest tissue for UPR gene expression analysis, transgenic plants overexpressing MfSTMIR, RNAi and *M. truncatula* Tnt1 MfSTMIR seedlings were grown in Fahraeus medium (*M. truncatula* handbook, Appendix 2 http://www.noble.org/MedicagoHandbook/) without Tm for 3 days, and then transferred to new medium with or without 10 μg ml⁻¹ Tm for 2 days. As a mock for the Tm treatment, an equivalent volume of DMSO was used in the same experimental procedure. The transcript levels of MtBiP1/2 and MtBIP3 in three biological replicates were quantified using qRT-PCR using a CFX96 Real-Time PCR Detection System (Bio-Rad, Berkeley, CA, USA) and the Mtactin was used as an endogenous control (Duan et al., 2017).

For Tm treatment of transgenic Arabidopsis plants overexpressing MfSTMIR, after germination on MS medium for 5 days, the seedlings were transferred to MS medium containing 0.5 μg ml⁻¹ Tm or DMSO for 8 days. The phenotype was photographed, and the percentage of green cotyledons was calculated. The values are presented as the mean ± standard deviation. Tests for significance were conducted using the non-parametric Kruskal–Wallis test using SPSS statistical software.

**Phylogenetic analysis**

To analyze the evolutionary relationships of MfSTMIR among different species, the MfSTMIR sequence, along with those from different species (*A. thaliana*, *M. truncatula*, *C. annuum*,...
C. arietinum, V. angularis, G. max, C. cajan) identified through the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov) database, and some reported TMD-RING E3 ubiquitin ligase sequences were used to construct a phylogenetic tree using MEGA software 5.05 using the neighbor-joining (NJ) method with 1000 bootstrap replicates.

**Tobacco infiltration assay**

Tobacco (N. benthamiana) infiltration assays were performed as described previously (Liu et al., 2010). For in vivo protein degradation experiments, Agrobacteria strains carrying constructs of MfSTMIR-HA, MfSTMIRm (H125A and H128A)-HA, substrate AtCPY* (G227R) and p19 genes, as well as internal control plasmids GFP-HA, were co-infiltrated at same tobacco leaves. Three days after infiltration, samples were collected and ground into powder with liquid nitrogen for protein gel blot assays. The MfSTMIR and mutants were prepared using overlap PCR-introduced site-directed mutagenesis.

For semi-in vivo protein degradation analysis, E3 and substrate were separately expression and extracted, then mixed and incubated at 4°C with gentle shaking. A final concentration of 10 μM ATP and 50 μM MG132 were separately added to the cell lysates to preserve or suppress the function of the 26S proteasome.

**Cellular fractionation and immunoblotting analysis**

To isolate the soluble cytoplasm and insoluble membrane, the 35S:MfSTMIR-FLAG fusion protein was transiently expressed in N. benthamiana and extracted as described previously (Lei et al., 2015). The protein concentrations from the soluble or insoluble fractions were measured using the Coomassie (Bradford) protein assay kit and were adjusted to equal concentration. The fractions (1 mg) were analyzed by 10% SDS-PAGE and then immunoblotted using Flag monoclonal antibody (F3165 Sigma). Next, a 1:5000 dilution of H-ATPase (Agrisera) and a 1:5000 dilution of cFPase (Agrisera) were used as plasma membrane and cytosolic markers, respectively.

**Subcellular localization**

To examine subcellular localization, the MfSTMIR ORF lacking a termination codon was inserted into the pE3025–GFP plasmid. RFP–HDEL served as a marker for ER localization. All the GFP/RFP fusion constructs were placed under the control of the 35S promoter and NOS terminator. Fluorescence signals were observed on a laser confocal microscope (Olympus Fluoview FX-PV1000).

**GUS bioassays**

To test the induction of GUS expression by salt and Tm, 4-week-old transgenic seedlings were transferred to Fahraeus medium with 1 μM NaCl or 10 μM mg l⁻¹ Tm for 12 h, the leaves were collected at 0, 6 and 12 h and stained at 37°C overnight in GUS staining solution to detect GUS expression.

**BiFC assays**

PCR-amplified coding regions of MfSTMIR, MtUBC32 and MtSec61yor were introduced into the Spel/Sal site. The resulting plasmid pSPYCE (M)-MfSTMIR contained the C-terminal (155 amino acid residues) region of eYFP, while pSPYNE(Ri)-MtUBC32 and pSPYNE(r)-MtSec61yor contained the N-terminal (173 amino acid residues) part of eYFP. VC and pSPYCE (M)-MfSTMIR129-70(R) were used as negative controls. BiFC was performed in 4-week-old Arabidopsis plants after polyethylene glycol (PEG)-mediated transient transformation, according to the method described by Shen’s laboratory protocol (Yoo et al., 2007).

**Firefly luciferase complementation imaging assays**

The method of firefly luciferase (Luc) complementation imaging and the vectors were described previously (Paulmurugan and Gambhir, 2007; Chen et al., 2008; MtUBC32 or MtSec61yor CDS was ligated to the CDS for the C-terminal end of split Luc in the 35S:CLuc vector between KpnI and SalI, yielding MtUBC32-CLuc or MtSec61yor-CLuc, respectively. MfSTMIR CDS was ligated to the CDS for the N-terminal end of split Luc in the 35S:Nluc vector between KpnI and SalI, yielding MfSTMIR-Nluc. The vectors were transformed into A. tumefaciens strain EHA105. Equal volumes of A. tumefaciens cultures harboring each of the Cluc and MfSTMIR-29-70-Nluc constructs were mixed to a final optical density at 600 nm of 1.0 in infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, and 200 mM acetoxyserine) and were infiltrated into fully expanded tobacco leaves using a 1-ml needleless syringe.

The agroinfiltrated tobacco plants were grown in the dark for 24 h and then exposed to a 15-h light/8-h dark cycle for 24 h at 23°C. Excess luciferin (560 μg ml⁻¹) (cat. no. E1602, Promega, Madison, WI, USA) was injected into the tobacco leaves. After 10 min, Luc activity was visualized using a CCD imaging apparatus (CHEMIPROHT 1300B/LND, 16 bits; Roper Scientific, Tucson, AZ, USA).

**In vitro self-ubiquitination assay**

Full-length MfSTMIR cDNA was PCR-amplified using specific primers (MfSTMIR Full F and MfSTMIR Full R in Table S1). The PCR product was digested with BamHI and SalI and then ligated into EcoRI-digested pMAG c2x (New England BioLabs, Ipswich, MA, USA). Recombinant MBP-MfSTMIR was expressed in Escherichia coli, purified by affinity chromatography using amylase resin (New England BioLabs), and then used for in vitro self-ubiquitination assays as described previously (Cho et al., 2008). Purified MBP-MfSTMIR (500 ng) was incubated in 30 μl of ubiquitination reaction buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂ along with 0.5 mM DTT, 4 mM ATP, 5 mg ubiquitin (Sigma-Aldrich, St. Louis, MO, USA), 100 ng of Human E1 (UBA1), and 100 ng of Rabbit E2 at 37°C for 2 h. The reaction products were separated by 10% SDS-PAGE and subjected to immunoblotting using anti-MBP antibody (New England Biolabs) or anti-ubiquitin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously (Lee et al., 2006).

**Split-ubiquitin yeast two-hybrid (suY2H) system**

Protein interactions were analyzed using the suY2H system (Ludwig et al., 2003; Obrdlik et al., 2004). Library screening was carried out using this system. The M. truncatula cDNA library was constructed by inserting cDNA into pPR3-N according to the manufacturer’s instructions (Dualsearch biotech p01001). The pBT3-STE or pPR3-N vectors contain a Leu⁺ or Trp⁺ marker, respectively. MfSTMIR was fused to Cub in pBT3-STE to generate pBT3-STE MfSTMIR-Cub as a bait vector. Yeast strain NMY51, containing HIS3, ADE2 and lacZ reporter genes, was used for library screening. To verify positive clones, prey plasmids were isolated from candidate positive colonies and then restested in yeast. Before library screening, pPR3-N and pBT3-STE MfSTMIR-Cub were co-transformed into NMY51, and a suitable 3-AT concentration was chosen to suppress background growth on synthetic dropout (SD)/—Ade—His—Leu—Trp medium. We chose 30 mg 3-AT for subsequent screening. To verify positive clones, prey plasmids were isolated from candidate positive colonies and then restested in yeast.
The MtUBC32 or MtSec61 coding sequence was PCR-amplified from M. truncatula cDNA. The amplified fragments were cloned into pPR3-N and verified by DNA sequencing. According to domain structure, MfSTMIR129-70, MfSTMIR171-104, and MfSTMIR105-150 were cloned into pBT3-STE. Interactions were tested in yeast strain NMY51 on SD/–Ade–His–Leu–Trp medium containing 30 mM 3-AT. Transformants were incubated at 30°C for 4 days. pPR3-N with pBT3-STE MfSTMIR-Cub was used a negative control. pAI-Alg5 and pBT3-STE MfSTMIR-Cub were used as positive controls.

**ACCESSION NUMBERS**

The sequence data from this article have been deposited in the NCBI database under the following accession numbers: MfSTMIR (XM_003601855.2), MtUBC32 (XM_003608715), AtCPY (NM_111876.5), MtBiP12 (XM_013591793.1), MtBiP9 (XM_013591901.1), MtSec61 (XM_003616720.2), MsSec61β (XM_003609992.2).

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**AUTHOR CONTRIBUTIONS**

TW and JD designed the research and revised the manuscript; RZ and HC performed the main experiments and analyzed the data together; MD participated in the stress treatments; FZ participated in the modification of some constructs; and JW provided the Tnt1 insertion mutant. All authors read and approved the final manuscript.

**CONFLICT OF INTEREST**

There are no conflicts of interest.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Cis-elements of the MfSTMIR promoter.

**Figure S2.** DNA and mRNA levels in MfSTMIR-overexpressing Medicago.

**Figure S3.** DNA and mRNA levels in MfSTMIR-RNAi Medicago.

**Figure S4.** Identification of NF7550, a Tnt1 insertion mutant of MfSTMIR.

**Figure S5.** DNA and mRNA levels in MfSTMIR-overexpressing Arabidopsis.

**Figure S6.** MfSTMIR facilitates the degradation of AtCPY–Nluc fusion protein.

**Figure S7.** Immunoblotting detected the expression of MfSTMIR::29-70–HA–Nluc and MfSTMIR::HA–Nluc fusion protein.

**Table S1.** The candidate proteins interacted with MfSTMIR were screened from the cDNA library of Medicago by split-ubiquitin yeast two-hybrid system.

**Table S2.** Gene-specific primers used for qRT-PCR, RT-PCR, and cloning.

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