

IRE1-mediated cytoplasmic splicing and regulated IRE1-dependent decay of mRNA in the liverwort *Marchantia polymorpha*

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Abstract The unfolded protein response (UPR) or the endoplasmic reticulum (ER) stress response is a homeostatic cellular response conserved in eukaryotes to alleviate the accumulation of unfolded proteins in the ER. In the present study, we characterized the UPR in the liverwort *Marchantia polymorpha* to obtain insights into the conservation and divergence of the UPR in the land plants. We demonstrate that the most conserved UPR transducer in eukaryotes, IRE1, is conserved in *M. polymorpha*, which harbors a single gene encoding IRE1. We showed that MpIRE1 mediates cytoplasmic splicing of mRNA encoding MpbZIP7, a *M. polymorpha* homolog of bZIP60 in flowering plants, and upregulation of ER chaperone genes in response to the ER stress inducer tunicamycin. We further showed that MpIRE1 also mediates downregulation of genes encoding secretory and membrane proteins in response to ER stress, indicating the conservation of regulated IRE1-dependent decay of mRNA. Consistent with their roles in the UPR, *Mpire1^{se}* and *Mpbzip7^{se}* mutants exhibited higher sensitivity to ER stress. Furthermore, an *Mpire1^{se}* mutant also exhibited retarded growth even without ER stress inducers, indicating the importance of MpIRE1 for vegetative growth in addition to alleviation of ER stress. The present study provides insights into the evolution of the UPR in land plants.

Key words: bZIP transcription factor, cytoplasmic splicing, endoplasmic reticulum stress, *Marchantia polymorpha*, unfolded protein response.

Introduction

The unfolded protein response (UPR) or the endoplasmic reticulum (ER) stress response occurs in eukaryotic cells when physiological and environmental conditions perturb protein folding and maturation in the ER causing accumulation of unfolded proteins, termed ER stress (Walter and Ron 2011). Although the UPR is widely conserved in eukaryotic cells, the molecular components of the UPR are diverged in many eukaryotes. The UPR was first described in the budding yeast *Saccharomyces cerevisiae* as a cellular response to accumulation of unfolded proteins in the ER, in which genes encoding the ER-resident molecular chaperones and folding enzymes are transcriptionally activated. ER stress is sensed by IRE1, an ER membrane-localized protein with an ER luminal domain and cytosolic kinase and ribonuclease

domains (Cox et al. 1993). IRE1 mediates cytoplasmic splicing of *Hac1* mRNA, which removes a 252-nt long intron, and the resulting *Hac1* mRNA encodes an active Hac1 bZIP transcription factor with a newly translated transactivation domain at the C-terminus (Cox and Walter 1996; Kawahara et al. 1998).

In contrast, animals harbor three arms of the UPR signaling pathway. IRE1-mediated cytoplasmic splicing is conserved in animals, in which a 26-nt intron in *Xbp1* mRNA is removed, and the resulting *Xbp1* mRNA encodes an active Xbp1 bZIP transcription factor with a transactivation domain at its C-terminus that induces transcription of ER chaperone genes (Yoshida et al. 2001). IRE1 also mediates destabilization of mRNA encoding secretory and membrane proteins under ER stress, termed regulated IRE1-dependent decay (RIDD), to reduce newly synthesized proteins into the

Abbreviations: ER, endoplasmic reticulum; qRT-PCR, quantitative RT-PCR; RIDD, regulated IRE1-dependent decay; RIP, regulated intramembrane proteolysis; UPR, unfolded protein response.

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stressed ER (Hollien and Weissman 2006; Hollien et al. 2009). The second arm of the UPR is regulated intramembrane proteolysis (RIP)-activated ATF6 (Haze et al. 1999; Yoshida et al. 2001). ATF6 is synthesized as an ER membrane-anchored protein that is inactive as a transcription factor. ATF6 is cleaved within its transmembrane domain in response to ER stress, which releases the cytoplasmic portion of ATF6 including its bZIP domain that can translocate to the nucleus where it induces transcription of ER chaperone genes. The third arm of the UPR is PERK-mediated translational attenuation, in which an ER membrane kinase, PERK, phosphorylates eIF2 α and attenuates translation in response to ER stress, which alleviates ER stress by reducing newly synthesized proteins into the stressed ER (Harding et al. 1999, 2000).

Flowering plants harbor two arms of the UPR signaling pathway (Iwata and Koizumi 2012). One arm consists of the most conserved ER stress sensor IRE1, which mediates cytoplasmic splicing of *bZIP60* mRNA and RIDD (Deng et al. 2011; Mishiba et al. 2013; Nagashima et al. 2011). Cytoplasmic splicing in plants was first reported in *Arabidopsis bZIP60*, and was experimentally verified also in other flowering plants such as rice, maize, and *Brachypodium* (Hayashi et al. 2012; Kim et al. 2017; Li et al. 2012). *bZIP60* mRNA encodes a bZIP domain followed by a transmembrane domain, which anchors bZIP60 proteins on the ER membrane. Cytoplasmic splicing of *bZIP60* mRNA removes a 23-nt long intron and causes a frameshift, resulting in the translation of a new ORF that does not encode a transmembrane domain and the production of nuclear-localized bZIP60 (Deng et al. 2011; Nagashima et al. 2011). Another UPR arm consists of an ATF6-like membrane-bound bZIP transcription factor that is activated by RIP. RIP-regulated bZIP28 (Che et al. 2010; Kim et al. 2018; Liu et al. 2007; Tajima et al. 2008) was first identified in *Arabidopsis*, and the rice homolog OsbZIP39 (Takahashi et al. 2012) was also reported to be activated by ER stress and functions as a transcription factor. In contrast, PERK-mediated translational attenuation has been considered to be absent in plants, unlike animal systems.

In contrast to the UPR in flowering plants, experimental evidence for the UPR in basal land plant lineages is lacking. The present study was aimed to obtain insights into the conservation and divergence of the UPR in the land plants by characterization of the UPR in the liverwort *Marchantia polymorpha*, one of the basal-most land plant lineages.

Materials and methods

Plant materials and stress treatment

M. polymorpha Tak-1 and its mutants were used in the present

study. They were grown on half-strength B5 medium at 25°C in 16-h light/8-h dark cycle. Tak-1 and mutant gemmae were grown on half-strength B5 medium for 4 weeks, and thalli were immersed on half-strength B5 medium containing 5 $\mu\text{g ml}^{-1}$ tunicamycin or 0.1% dimethyl sulfoxide as a solvent control for indicated time periods for RNA extraction. For ER stress sensitivity assay, Tak-1 and mutant gemmae were grown on half-strength B5 medium containing indicated concentrations of tunicamycin or dithiothreitol for 4 weeks, and photographed.

RNA analysis

Total RNA was extracted from thalli using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 2006). The extracted RNA was treated with DNase (Nippongene, Tokyo, Japan) and reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts) with random hexamers. To detect different *MpbZIP7* mRNA isoforms, PCR was performed with primers that amplify both *MpbZIP7u* and *MpbZIP7s*. PCR products were fractionated on 8% polyacrylamide gel and detected by ethidium bromide staining. For quantification, cDNA was subjected to quantitative real-time PCR by using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) with 7300 Real-Time PCR System (Applied Biosystems, California). The mRNA level was normalized against *MpEF1-a* levels. Primers used are listed in Supplementary Table S1.

Generation of *M. polymorpha* mutants

Oligonucleotide sequences for gRNAs used are listed in Supplementary Table S1. The oligonucleotides were annealed and the resulting dsDNA with 5'-protruding ends was ligated to pMpGE-En03 (Sugano et al. 2018) that had been digested with *BsaI* (New England Biolabs, Massachusetts). The resulting vectors were introduced into pMpGE010 (Sugano et al. 2018) by using LR Clonase II (Thermo Fisher Scientific, Massachusetts).

These vectors were introduced into *Rhizobium radiobacter* strain GV2260. Liverwort transformation was carried out according to the previously reported protocol using thalli (Ishizaki et al. 2008; Kubota et al. 2013). Transformants were selected against half-strength B5 medium containing 100 mg l^{-1} claforan and 10 mg l^{-1} hygromycin or 50 μM chlorsulfuron.

Results and discussion

ER chaperone genes in *Marchantia*

We first searched the *M. polymorpha* genome sequence for genes encoding ER chaperones, BiP, CNX and CRT. We found that the *M. polymorpha* genome encodes single gene for each chaperone. This is in sharp contrast with the case of flowering plants, because flowering plants genomes tend to encode multiple genes for each chaperone. For instance, the *Arabidopsis thaliana* genome encodes three *BiP*, two *CNX*, and three *CRT* genes (Iwata et al. 2008). We investigated whether these ER chaperone

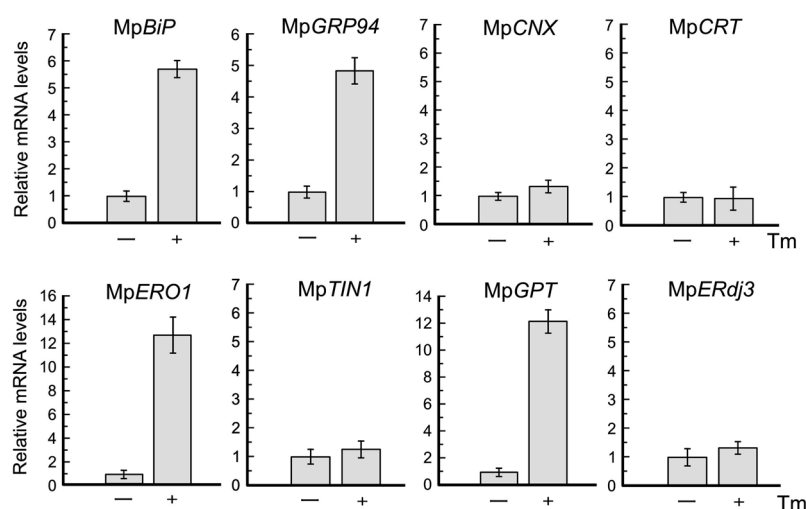


Figure 1. Induction of ER chaperone genes in *M. polymorpha*. qRT-PCR analysis in response to tunicamycin treatment. Thalli of Tak-1 grown on B5 medium for 4 weeks were immersed in liquid B5 medium containing $5 \mu\text{g ml}^{-1}$ tunicamycin (+Tm) or 0.1% dimethyl sulfoxide (–Tm), incubated for 5 h, and subjected to qRT-PCR analysis to quantify the expression of the indicated genes. *MpEF1-a* was used as a control. The data are means with SE from three independent biological replicates.

genes are transcriptionally responsive to ER stress. We treated thalli of Tak-1 with an ER stress inducer, tunicamycin, which inhibits *N*-glycosylation of proteins synthesized in the ER, and subjected to qRT-PCR. As shown in Figure 1, *MpBiP* was induced by tunicamycin treatment, whereas *MpCRT* and *MpCNX* transcript levels were unchanged. We also tested other genes related to ER protein quality control. The data showed that *MpGRP94*, *MpERO1*, and *MpGPT* genes are induced by tunicamycin treatment as in the case of *Arabidopsis* homologs (Iwata et al. 2008, 2010), whereas transcript levels for *MpTIN1* and *MpERdj3*, whose homologs in *Arabidopsis* are responsive to tunicamycin (Iwata et al. 2012; Yamamoto et al. 2008), are unchanged. It indicates that although the transcriptional induction system of the UPR is conserved in *M. polymorpha*, a limited set of ER chaperone genes are the target of the UPR compared to flowering plants.

Cytoplasmic splicing of *bZIP60* homologs in *Marchantia*

We next searched *bZIP* genes in liverwort for homologous genes encoding *bZIP60*, a cytoplasmic splicing target of IRE1 (Deng et al. 2011; Nagashima et al. 2011). The unspliced forms (u forms) of *Arabidopsis bZIP60* (Deng et al. 2011; Nagashima et al. 2011) and rice *bZIP50* (Hayashi et al. 2012) encode a transmembrane domain, and a new ORF is translated from the spliced forms (s forms) that do not encode a transmembrane domain (Figure 2A). The u forms of *Arabidopsis bZIP60* and rice *bZIP50* mRNAs harbor two stem-loops, where the cleavage occurs between third and fourth nucleotides of the loops (Figure 2B). Among 15 genes encoding *bZIP* domain in the *M. polymorpha* genome (*MpbZIP1* – *MpbZIP15*), *MpbZIP7* fulfilled such criteria (Figure 2A, B, Supplementary Figure S1). The predicted intron

of *MpbZIP7* mRNA is 23 nucleotides in length, which causes a frameshift as in the case of flowering plant *bZIP60* and animal *XBP1* but different from budding yeast *Hac1* (Deng et al. 2011; Kawahara et al. 1998; Nagashima et al. 2011; Sidrauski et al. 1996; Yoshida et al. 2001). Furthermore, the first C, the third G, and the sixth G in the first and second loops of *Arabidopsis bZIP60* mRNA have been shown to be critical for cleavage by IRE1, and the corresponding nucleotides were conserved in *MpbZIP7* mRNA as well (Figure 2B).

We examined whether cytoplasmic splicing of *MpbZIP7* mRNA indeed occurs. We treated thalli of Tak-1 with tunicamycin and subjected them to RT-PCR using primers that amplify an *MpbZIP7* cDNA region that contains the predicted intron. As shown in Figure 2C, in addition to a signal detected from mock-treated thalli, an additional faster-migrating band was detected from tunicamycin-treated thalli. This faster-migrating signal was detected in the course of tunicamycin treatment (Figure 2D). To confirm whether this additional signal is indeed derived from the spliced *MpbZIP7* mRNA, we cloned and sequenced both fragments. As shown in Figure 2E, these bands corresponded to u and s forms of *MpbZIP7*. These data show that the cytoplasmic splicing of *MpbZIP7* mRNA occurs in response to ER stress, removing the 23-nucleotide intron.

Cytoplasmic splicing of *MpbZIP7* by *MpIRE1*

Cytoplasmic splicing of *bZIP* mRNA in eukaryotes is mediated by an ER-localized ribonuclease, IRE1. A BLAST search showed that *M. polymorpha* carries one *IRE1* gene (Figure 3A, Supplementary Figure S1). *MpIRE1* harbors an N-terminal signal peptide, a transmembrane domain, a kinase domain, and a ribonuclease domain, which are characteristics of

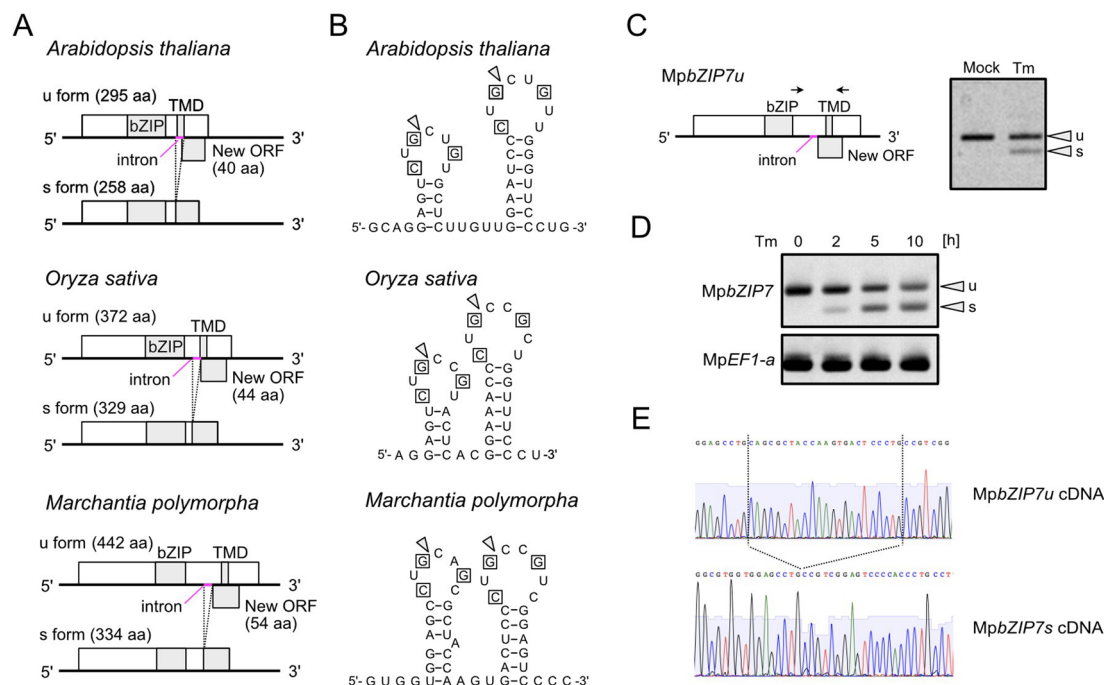


Figure 2. Cytoplasmic splicing of *MpbZIP7*. (A) Diagram of mRNA for *A. thaliana* *bZIP60*, *O. sativa* *bZIP50*, and *M. polymorpha* *bZIP7*. bZIP domain (bZIP), transmembrane domain (TMD), and new ORF translated after cytoplasmic splicing are indicated. Cytoplasmic splicing removes an intron of 23 nt (*A. thaliana* and *M. polymorpha*) or 20 nt (*O. sativa*) from unspliced mRNA (u form), and the new ORF is translated from the resulting spliced mRNA (s form). (B) Predicted secondary structure around the splice site of mRNA. Arrowheads indicate predicted cleavage positions by IRE1. Squares indicate the conserved nucleotides required for cytoplasmic splicing in plants, animals and yeast. (C) Detection of unspliced and spliced forms of *MpbZIP7*. Four-week-old thalli were treated with $5 \mu\text{g ml}^{-1}$ tunicamycin (Tm) or 0.1% dimethyl sulfoxide (Mock) for 5 h and subjected to RT-PCR. Primers used to amplify both unspliced (u) and spliced (s) forms are indicated by arrows. Arrowheads on the right of the gel image indicate u and s forms. (D) Time course analysis of *MpbZIP7* splicing. Four-week-old thalli were treated with $5 \mu\text{g ml}^{-1}$ tunicamycin (Tm) at indicated periods and subjected to RT-PCR as in C. (E) Sanger sequencing analysis of spliced *MpbZIP7*. PCR products corresponding to u and s forms in C were cloned into pGEM-T Easy vector and subjected to Sanger sequencing.

canonical IRE1 proteins (Figure 3A).

We next generated *Mpire1^{se}* mutants by CRISPR/Cas9 system (^{se} indicates mutants generated by genome editing) and tested whether *MpbZIP7* mRNA splicing is dependent on MpIRE1. We obtained five *Mpire1^{se}* mutants, two of which harbor an identical one base deletion mutation (Figure 3A). We then tested these mutants along with Tak-1 with RT-PCR that amplifies both u and s forms of *MpbZIP7*. As shown in Figure 3B, *MpbZIP7s* was undetected in all *Mpire1^{se}* mutants even under ER stress conditions. It indicates that *MpbZIP7* splicing is dependent on MpIRE1.

Cytoplasmic splicing of *Arabidopsis bZIP60u* mRNA is considered to require the transmembrane domain of *bZIP60u* because the transmembrane domain brings *bZIP60u* mRNA to the ER membrane where IRE1 cleavage occurs (Deng et al. 2011; Nagashima et al. 2011). If this is also the case with *MpbZIP7*, a frameshift mutation before the transmembrane domain-coding region of *MpbZIP7u* would escape *MpbZIP7u* mRNA from splicing. To test this, we generated *Mpbzip7^{se}* mutants by CRISPR/Cas9 system. Among three gRNAs tested (gRNA1–3), we obtained mutants with gRNA2 and gRNA3 (Figure 3C). These *Mpbzip7^{se}* mutants harbor

1-base and 7-base deletions, respectively, and therefore the resulting mutant *MpbZIP7u* proteins do not contain a transmembrane domain. We examined whether *MpbZIP7u* undergoes cytoplasmic splicing in the *Mpbzip7^{se}* mutants. As shown in Figure 3D, *MpbZIP7s* was undetected in *Mpbzip7^{se}* mutants even under ER stress conditions. It suggests that the cytoplasmic splicing requires the transmembrane domain of *MpbZIP7u* for ER localization of *MpbZIP7u* mRNA for the IRE1 cleavage.

MpIRE1-dependent upregulation of ER chaperone genes and downregulation of mRNA encoding a secretory pathway protein

We next asked whether upregulation of ER chaperone genes in response to ER stress is dependent on the MpIRE1/*MpbZIP7* pathway. Induction of the *MpBiP* gene in response to tunicamycin was significantly reduced in all *Mpbzip7^{se}* and *Mpire1^{se}* mutants (Figure 4A). Induction of other UPR target genes, *MpGRP94*, *MpERO1*, and *MpGPT*, was abolished in *Mpbzip7^{se}* and *Mpire1^{se}* mutants (Figure 4B). Induction of *MpERdj3*, *MpTIN1*, and *MpPDI*, whose induction by tunicamycin treatment was undetected in Tak-1, was not observed in

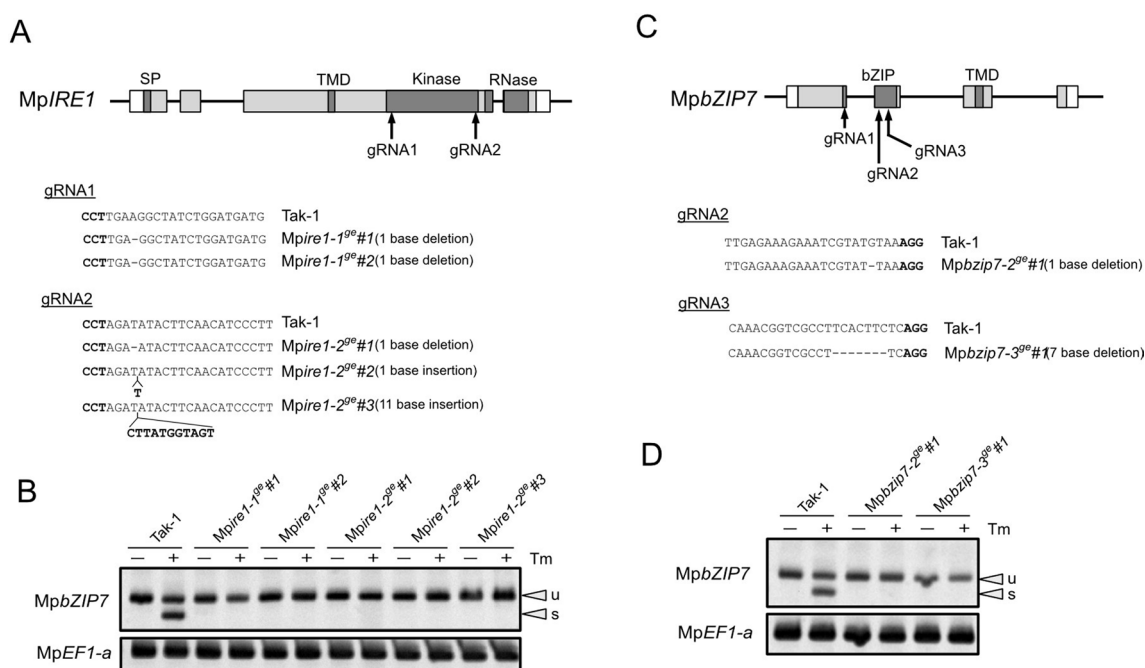


Figure 3. Effect of mutations in MpIRE1 and MpbZIP7 on cytoplasmic splicing. (A) Generation of *Mpire1^{se}* mutants. The MpIRE1 gene structure and the nucleotide sequences of the generated mutants are shown. An open box indicates an untranslated region. Signal peptide (SP), transmembrane domain (TMD), kinase domain, and RNase domain are also indicated. Locations of gRNA target sites are indicated in the diagram. PAM sequences are shown in bold. (B) MpbZIP7 splicing in *Mpire1^{se}* mutants. Tak-1 and *Mpire1^{se}* mutants were treated with 5 $\mu\text{g ml}^{-1}$ tunicamycin (+Tm) or 0.1% dimethyl sulfoxide (–Tm) and subjected to RT-PCR analysis as in Figure 2C. Arrowhead on the right of the gel image indicate unspliced (u) and spliced (s) forms of MpbZIP7. MpEF1-a was analyzed as a control. (C) Generation of *Mpbzip7^{se}* mutants. The MpbZIP7 gene structure and the nucleotide sequences of the generated mutants are shown. bZIP domain (bZIP) and transmembrane domain (TMD) are also indicated. A location of gRNA target site is indicated in the diagram. PAM sequence is shown in bold. (D) MpbZIP7 splicing in *Mpbzip7^{se}* mutants. Tak-1 and *Mpbzip7^{se}* mutants were treated with 5 $\mu\text{g ml}^{-1}$ tunicamycin (+Tm) or 0.1% dimethyl sulfoxide (–Tm) and subjected to RT-PCR analysis as in B. Arrowhead on the right of the gel image indicate unspliced (u) and spliced (s) forms of MpbZIP7.

both *Mpbzip7^{se}* and *Mpire1^{se}* (Figure 4B). It should be noted that the transcript levels of MpTIN1 and MpPDI were decreased in *Mpbzip7^{se}*, but further experiments would be needed to elucidate the underlying mechanism.

We next investigated whether IRE1-dependent destabilization of mRNA encoding secretory and membrane proteins, termed RIDD, is conserved in *M. polymorpha*. We chose three genes that encode a signal peptide at their N-terminus and two genes that do not, and quantified their mRNA levels in response to ER stress. A decrease in mRNA level of genes encoding a signal peptide was observed in Tak-1, and this was abolished in *Mpire1^{se}* but not in *Mpbzip7^{se}* (Figure 4C). In contrast, expression of two genes that do not encode a signal peptide, was unchanged by tunicamycin treatment among Tak-1 and the mutants (Figure 4C). These data indicate that downregulation of mRNA encoding a secretory protein under ER stress is conserved in *M. polymorpha*.

Although IRE1 is the most conserved ER stress transducer in eukaryotes, a functional diversification of IRE1 has been observed. It has been demonstrated that RIDD function of IRE1 is conserved in animals and flowering plants (Hollien and Weissman 2006; Hollien et al. 2009; Mishiba et al. 2013) but not in the budding

yeast *S. cerevisiae*. Furthermore, IRE1 from certain species is able to mediate RIDD but lacks cytoplasmic splicing target mRNA. For instance, IRE1 from the fission yeast *Schizosaccharomyces pombe* mediates RIDD but apparently does not cleave any mRNA encoding a transcription factor (Kimmig et al. 2012), indicating that ER stress is alleviated through mRNA degradation without transcriptional induction in certain species. The present study demonstrated that RIDD is conserved in *M. polymorpha* IRE1 as in flowering plants and suggested that IRE1 has maintained RIDD function through the land plant evolution.

Our BLAST search found that MpbZIP14 also encodes a transmembrane domain following a bZIP domain as does Arabidopsis bZIP28, another bZIP transcription factor. Therefore, *M. polymorpha* also harbors a RIP-regulated UPR activation mechanism in addition to cytoplasmic splicing and RIDD. RIP-regulated bZIP transcription factors in animals and flowering plants have been shown to be activated by S2P, a Golgi membrane-localized metalloprotease (Che et al. 2010; Iwata et al. 2017; Ye et al. 2000). Because our BLAST search also found one S2P homolog in the *M. polymorpha* genome, it is likely that MpS2P also cleaves and activates MpbZIP14. Involvement of MpbZIP14 and MpS2P in the *M.*

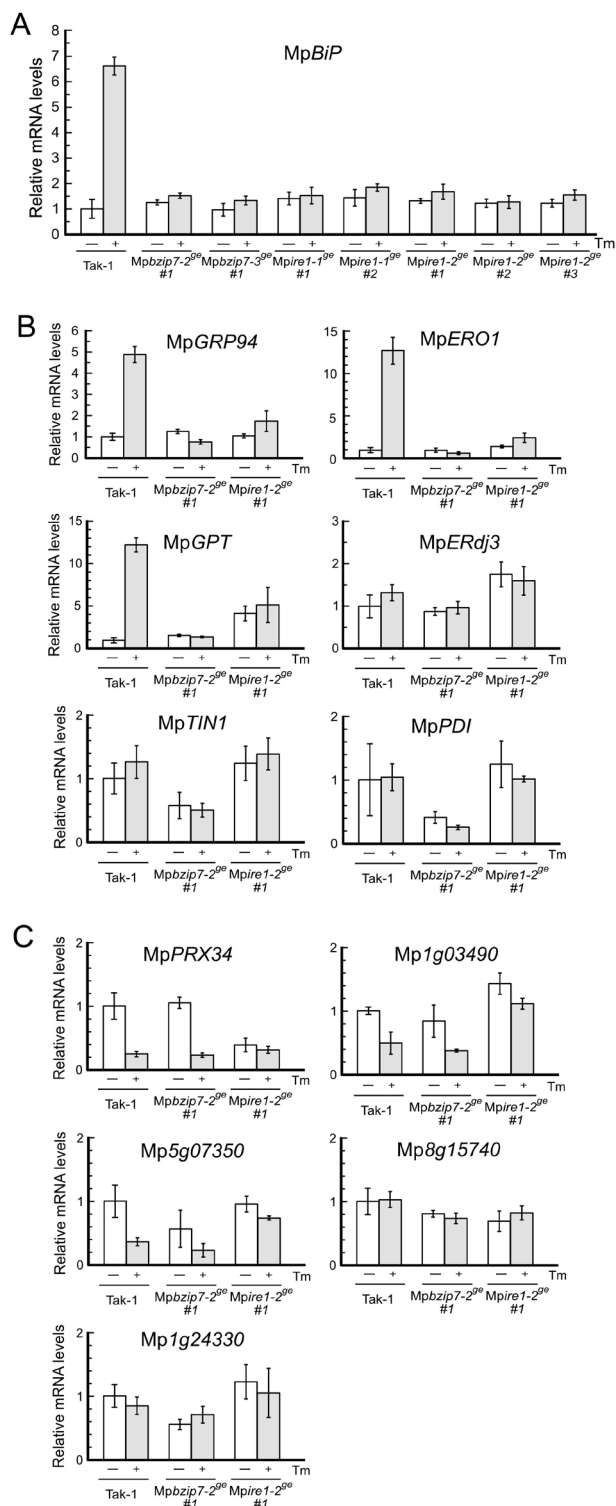


Figure 4. Expression analysis of UPR-target genes in *Mpbzip7^{8e}* and *Mpire1^{8e}* mutants. (A) qRT-PCR analysis of *MpBiP* in *Mpbzip7^{8e}* and *Mpire1^{8e}* mutants. Tak-1 and the indicated mutants were treated with 5 μ M tunicamycin (+Tm) or 0.1% dimethyl sulfoxide (–Tm) for 5 h and subjected to qRT-PCR analysis. *MpEF1-a* was used as a control. The data are means with SE from three independent biological replicates. (B) qRT-PCR analysis of other ER chaperone genes in *Mpbzip7^{8e}* and *Mpire1^{8e}* mutants. qRT-PCR was carried out as in A. (C) qRT-PCR analysis of mRNA encoding proteins destined to the secretory pathway in *Mpbzip7^{8e}* and *Mpire1^{8e}* mutants. qRT-PCR was carried out as in A. Three genes that encode an N-terminal signal peptide (*MpPRX34*, *Mp1g03490*, and *Mp5g07350*) and two genes that do not (*Mp8g15740* and *Mp1g24330*) were analyzed.

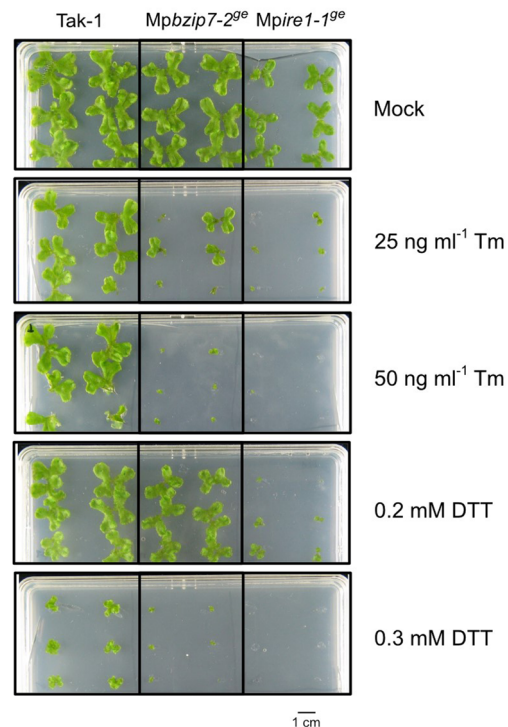


Figure 5. Growth of *Mpbzip7^{8e}* and *Mpire1^{8e}* mutants. Gemmae of Tak-1 and the indicated mutants were inoculated on half-strength B5 medium containing the indicated concentration of tunicamycin (Tm) or dithiothreitol (DTT), grown for 20 days, and photographed.

polymorpha UPR await experimental verification.

In contrast to the widely conserved *IRE1* gene, it has been known that *PERK* is present in animals but absent in yeast and flowering plants. Our BLAST search in the *M. polymorpha* genome also did not detect a homologous gene for *PERK*. It suggests that *PERK* has emerged and acquired its translational control function through animal evolution.

ER stress sensitivity of *Mpire1^{8e}* and *Mpbzip7^{8e}* mutants

We next examined the effect of mutations in UPR components on ER stress sensitivity in *M. polymorpha*. We grew Tak-1 and *Mpire1^{8e}* and *Mpbzip7^{8e}* mutants on half-strength B5 medium containing ER stress inducers, tunicamycin and dithiothreitol, and observed their growth. As shown in Figure 5, both *Mpire1^{8e}* and *Mpbzip7^{8e}* mutants exhibited hypersensitivity to tunicamycin and dithiothreitol. ER stress hypersensitivity was observed in *Mpbzip7^{8e}*, indicating cytoplasmic splicing is important for ER stress tolerance. Because the ER stress hypersensitivity was more pronounced in *Mpire1^{8e}* than in *Mpbzip7^{8e}*, *RIDD* is also important for mitigating ER stress.

Interestingly, growth retardation of *Mpire1^{8e}* was observed even without ER stress inducers, whereas *Mpbzip7^{8e}* grew normally as did Tak-1 (Figure 5), indicating the role of *MpIRE1*, presumably *RIDD*

activity of MpIRE1, in vegetative growth. In *Arabidopsis*, IRE1 is essential for normal growth and development because mutations of all the three *IRE1* genes cause lethality (Mishiba et al. 2019). Although not essential, the present study indicates that IRE1 is important for vegetative growth in *M. polymorpha*. It is also plausible that *M. polymorpha* IRE1 has functions in development and function of gametophytes such as sperm, because *Arabidopsis* IRE1 is important for functions of male gametophytes (Deng et al. 2013; Mishiba et al. 2019; Pu et al. 2019).

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