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 and Mpzbp7 mutants exhibited higher sensitivity to ER stress. Furthermore, an Mpire1 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 newly translated 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 eIF2α 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 RIP. RIP-regulated ATF6 is known to function 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

*Marchantia 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 µg 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 *Marchantia 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). These vectors were introduced into *Rhizobium radiobactor* 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 clorafon and 10 mg l⁻¹ hygromycin or 50 µM chlorosulfuron.

Results and discussion

ER chaperone genes in Marchantia

We first searched the *Marchantia polymorpha* genome sequence for genes encoding ER chaperones, BiP, CNX and CRT. We found that the *Marchantia 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...
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 MplIRE1

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). MplIRE1 harbors an N-terminal signal peptide, a transmembrane domain, a kinase domain, and a ribonuclease domain, which are characteristics of
We next generated Mpire1\textsuperscript{ge} mutants by CRISPR/Cas9 system (\textit{ge} indicates mutants generated by genome editing) and tested whether MpZIP7 mRNA splicing is dependent on MpIRE1. We obtained five Mpire1\textsuperscript{ge} 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 \textit{u} and \textit{s} forms of MpZIP7. As shown in Figure 3B, MpZIP7\textit{s} was undetected in all Mpire1\textsuperscript{ge} mutants even under ER stress conditions. It indicates that MpZIP7 splicing is dependent on MpIRE1.

Cytoplasmic splicing of Arabidopsis \textit{bZIP60u} mRNA is considered to require the transmembrane domain of \textit{bZIP60u} because the transmembrane domain brings \textit{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 MpZIP7, a frameshift mutation before the transmembrane domain-coding region of MpZIP7\textit{u} would escape MpZIP7\textit{u} mRNA from splicing. To test this, we generated Mpbzip7\textsuperscript{ge} mutants by CRISPR/Cas9 system. Among three gRNAs tested (gRNA1–3), we obtained mutants with gRNA2 and gRNA3 (Figure 3C). These Mpbzip7\textsuperscript{ge} mutants harbor 1-base and 7-base deletions, respectively, and therefore the resulting mutant MpbZIP7\textit{u} proteins do not contain a transmembrane domain. We examined whether MpbZIP7\textit{u} undergoes cytoplasmic splicing in the Mpbzip7\textsuperscript{ge} mutants. As shown in Figure 3D, MpbZIP7\textit{s} was undetected in Mpbzip7\textsuperscript{ge} mutants even under ER stress conditions. It suggests that the cytoplasmic splicing requires the transmembrane domain of MpbZIP7\textit{u} for ER localization of MpbZIP7\textit{u} 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/MpZIP7 pathway. Induction of the Mp\textit{BiP} gene in response to tunicamycin was significantly reduced in all Mpbzip7\textsuperscript{ge} and Mpire1\textsuperscript{ge} mutants (Figure 4A). Induction of other UPR target genes, Mp\textit{GRP94}, Mp\textit{ERO1}, and Mp\textit{GPT}, was abolished in Mpbzip7\textsuperscript{ge} and Mpire1\textsuperscript{ge} mutants (Figure 4B). Induction of Mp\textit{ERdj3}, Mp\textit{TIN1}, and Mp\textit{PDI}, whose induction by tunicamycin treatment was undetected in Tak-1, was not observed in
both $Mp_{bzip7}$ and $Mp_{ire1}$ (Figure 4B). It should be noted that the transcript levels of $Mp_{TIN1}$ and $Mp_{PDI}$ were decreased in $Mp_{bzip7}$, 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 $Mp_{ire1}$ but not in $Mp_{bzip7}$ (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 $Mp_{bZIP14}$ 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 $Mp_{S2P}$ also cleaves and activates $Mp_{bZIP14}$. Involvement of $Mp_{bZIP14}$ and $Mp_{S2P}$ in the $M.$
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^ge^ and Mpbzip7^ge^ mutants**

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

Interestingly, growth retardation of Mpire1^ge^ was observed even without ER stress inducers, whereas Mpbzip7^ge^ 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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