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Short Communication

Visualization of endoplasmic reticulum stressed cells for forward genetic studies in plants

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ABSTRACT

In eukaryotes, various cellular events are attended by a risk of triggering stress in the endoplasmic reticulum (ER). Such risks are assumed to be minimized by sophisticated regulation systems, but the nature of these systems remains largely unknown in plants. Here, transgenic *Arabidopsis* plants, intended for use in forward genetic studies of plant ER stress, are described. *AtBiP3* promoter activity clearly reflected the effects of inducers of ER stress, such as tunicamycin, dithiothreitol, and salicylic acid. Thus transgenic plants, containing the *AtBiP3* promoter coupled to a fluorescent protein-encoding gene, were generated to enable visual detection of cells experiencing ER stress in living plants. Mutagenization of these transgenic plants produced seedlings which exhibited altered fluorescence patterns. Constitutive fluorescence was observed in a number of independent lines, suggesting the plant genome includes many genes whose mutation results in ER stress. Some mutants showed strong fluorescence with different tissue specificity, implying potential ER stresses in individual cellular events. These results indicate that forward genetic approaches will provide useful information in the understanding of ER stress.

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Introduction

The endoplasmic reticulum (ER) is an important organelle in eukaryotes. Most proteins are synthesized in ribosomes on the rough ER, and a number are matured in the ER lumen. The accumulation of unfolded and misfolded proteins in the lumen disrupts ER homeostasis and is called "ER stress". Many eukaryotes have a response system, the "unfolded protein response" (UPR), which relieves ER stress (Walter and Ron, 2011).

In many eukaryotes, ER stress is sensed by IRE1, an ER transmembrane protein (Koizumi et al., 2001; Hetz and Glimcher, 2009; Walter and Ron, 2011). Activation of IRE1 by ER stress induces the unconventional splicing of mRNAs encoding transcription factors, such as HAC1 (yeast), XBP1 (animals), AtbZIP60 (*Arabidopsis*), and OsbZIP50 (rice), leading to a transition to their active forms as a result of frame shifting (Mori et al., 2000; Yoshida et al., 2001; Deng et al., 2011; Nagashima et al., 2011; Hayashi et al., 2012a). Representative targets of these transcription factors are genes encoding ER chaperone proteins, such as BiP.

UPR is activated by treatments that disrupt protein folding, such as high temperature or the addition of reducing agents (*e.g.*, dithiothreitol) or inhibitors of glycosylation (*e.g.*, tunicamycin). In addition, exogenous salicylic acid (SA) induces UPR *via* an unknown mechanism in *Arabidopsis* (Lu et al., 2012; Moreno et al., 2012; Nagashima et al., 2014), although it also serves as an effective suppressor of UPR in rice (Hayashi et al., 2012b). Since SA is a signaling molecule which plays a central role in plant defense and disease resistance, its effect on UPR is a major topic in plant science.

It is very important to know when and where the risk of ER stress is increased and how it is minimized by plant cells. Such knowledge is crucial for the improvement of technologies using plant cells as a platform for the production of recombinant proteins. We succeeded in accumulating high amounts of transgenic products in the endosperm of rice seeds by means of a protein storage pathway (Takaiwa et al., 2007) but the production of transgenic secretory proteins sometimes caused severe ER stress and degraded seed quality (Oono et al., 2010; Wakasa et al., 2012).

Plants have unique features such as dynamic organ formation and sophisticated environmental responses. There are likely to be many differences between the ER stress-related cellular systems of plants and other eukaryotes but, although the reverse genetic approach has been a mainstay of research into plant ER stress responses, the extent to which plant responses differ from those of other eukaryotes remains insufficiently understood.

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We generated transgenic *Arabidopsis* plants to enable visual detection of ER-stressed cells in living plant tissues. Additionally, using these transgenic plants, we examined the usefulness of the forward genetic approach in the investigation of ER stress.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh., Columbia (Col) ecotype, was used throughout this study. Plants were grown on MS plates (1× Murashige and Skoog salt mix, 2% sucrose, 2.5 mM MES (pH 5.8) and 0.8% agar) or on soil–vermiculite mixture at 23 °C under 16 h light/8 h dark cycles. All seeds were first stratified at 4 °C for 2 d before transfer to a growth chamber. For reverse transcription (RT)-polymerase chain reaction (PCR) analysis, 10-d-old seedlings were transferred to MS plates containing 5 μg/ml tunicamycin (Tm), 2 mM dithiothreitol (DTT), 0.5 mM SA (sodium salicylate), or 2 mM 4-phenylbutyrate (4-PBA). As a negative control for Tm, an equal volume of dimethyl sulfoxide (final concentration of 0.1%) was added.

Oligonucleotides

Oligonucleotides used in this study are listed in Table S1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.03.008.

RNA extraction and gene expression analysis

Total RNA was extracted from root tissues using an RNeasy Mini Kit (QIAGEN) unless otherwise noted. First-strand cDNA was synthesized from 0.4 μg of total RNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO), which includes both oligo(dT) and random hexamers, according to the manufacturer's instructions. All RT-PCR analyses were performed using 1/80 of the prepared cDNA. Quantitative and semi-quantitative PCR were performed using SYBR® *Premix Ex Taq*™ (Takara) and TaKaRa *Ex Taq*™, respectively. For quantitative RT-PCR, relative quantification was achieved using a standard curve. The *AtACT8* gene was used as an internal reference.

Generation of transgenic plants

For the *AtBiP3* promoter, the upstream region (−715 to −1 relative to the start codon) of the *AtBiP3* gene was amplified from genomic DNA using PCR. The fragment was fused to the *GAL4-VP16* gene using PCR and then cloned into the pDONR221 Gateway entry vector (Invitrogen) *via* the BP reaction. The *Nos* terminator cloned into the pKS2-3 Gateway entry vector was used as the terminator of the *GAL4-VP16* gene. A tandemly repeated upstream activation sequence (UAS), the *DsRED2* gene, and the *Nos* terminator were fused together using PCR and then cloned into pKS4-1 Gateway entry vector *via* a ligation reaction. Fragments from these three entry vectors were linked and inserted into modified pHm43GW (Wakasa et al., 2006) using Multisite Gateway Technology (Invitrogen). Transgenic *Arabidopsis* plants were generated using the *Agrobacterium*-mediated floral-dip transformation method.

Mutant screening

Approximately 2000 seeds from the transgenic lines were treated with 0.1% ethyl methanesulfonate (EMS) for 16 h at room temperature and then washed with water. The seeds were stratified for 2 d and then sown in soil-filled pots. M2 generation seeds were separately harvested from 98 pots and formed independent pools. For mutant screening, approximately 400 M2 seeds from each pool were grown on 150 mm MS plates.

Fluorescence microscopy

Fluorescence microscopy was performed using a stereomicroscope (SZX12; Olympus) equipped with a mercury lamp and a filter set for detecting red fluorescent proteins.

Results and discussion

Design of the reporter system

To detect ER-stressed cells in living plants, we generated transgenic plants in which reporter proteins accumulated in response to ER stress. Such responsivity can be conferred by promoters of ER stress-responsive genes. As a candidate for such a gene, we first evaluated the expression pattern of *AtBiP3* (*At1g09080*), which is a representative ER stress-inducible gene encoding a BiP protein in *Arabidopsis* (Martínez and Chrispeels, 2003). Quantitative RT-PCR analysis indicated that *AtBiP3* transcript was barely detectable in root tissues in the absence of ER stress treatments, whereas it was drastically induced by Tm treatment (Fig. 1A). The accumulation of *AtBiP3* transcript was clearly suppressed by 4-PBA, which serves as a chemical chaperone, suggesting that unfolded proteins generated by Tm treatment were a major cause of the induction (Fig. 1A). Similar effects of these treatments were also consistently observed in the accumulation of spliced forms of *AtbZIP60* transcripts (Fig. 1A), which encode a transcription factor contributing to *AtBiP3* expression during the UPR (Iwata et al., 2008). Similar results were obtained when DTT was used in place of Tm (Fig. 1B).

Consistent with previous reports (Lu et al., 2012; Moreno et al., 2012; Nagashima et al., 2014), SA treatment also induced transcription of *AtBiP3* and *AtbZIP60s*, although the induction levels were weak compared to the effects of Tm and DTT (Fig. 1C). It is noteworthy that induction by SA was suppressed by 4-PBA (Fig. 1C), suggesting that accumulation of unfolded proteins by SA treatment was one of the major causes of the induction. By contrast, a previous study in rice (*Oryza sativa*) reported that exogenous SA served as a suppressor of UPR by an unknown mechanism (Hayashi et al., 2012b) but it was unknown whether similar, suppressive effects of SA were found in *Arabidopsis*. As shown in Fig. 1A and B, in the presence of SA, Tm and DTT-induced expression of *AtBiP3* was indeed largely suppressed, suggesting that the suppressive effects of SA were conserved between rice (a monocot) and *Arabidopsis* (a dicot).

These results demonstrated that plants have a unique response system, as shown in Fig. 1D, clearly reflected by the expression of *AtBiP3*. The activity of the *AtBiP3* promoter was therefore considered suitable for a reporter construct. Consistent with its responsivity, putative *cis*-elements involved in the plant UPR, such as the ER stress response element (ERSE) (Liu and Howell, 2010) and the plant unfolded protein response element II (pUPRE-II) (Hayashi et al., 2013), were found in the upstream region of *AtBiP3* gene (Fig. 2A).

To detect the activation of UPR at the cellular level visually, a red fluorescent protein DsRED2 was selected as the reporter protein; this choice had the advantage of minimizing cell damage during fluorescence microscopy because of the relatively long wavelength of light exciting red fluorescent proteins. Additionally, the DsRED2 protein has a long half-life (Verkhusha et al., 2003), although its chromophore formation is not fast. The property was expected to contribute to facilitate fluorescent observations. Due to concern that the activity of *AtBiP3* promoter would not be strong enough to produce a visually detectable level of DsRED2, we used the

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