The Unfolded Protein Response Modulates a Phosphoinositide-Binding Protein through the IRE1-bZIP60 Pathway

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Phosphoinositides function as lipid signals in plant development and stress tolerance by binding with partner proteins. We previously reported that Arabidopsis (Arabidopsis thaliana) phosphoinositide-specific phospholipase C2 functions in the endoplasmic reticulum (ER) stress response. However, the underlying molecular mechanisms of how phosphoinositides act in the ER stress response remain elusive. Here, we report that a phosphoinositide-binding protein, SMALLER TRICHOMES WITH VARIABLE BRANCHES (SVB), is involved in the ER stress tolerance. SVB contains a DUF538 domain with unknown function; orthologs are exclusively found in Viridiplantae. We established that SVB is ubiquitously expressed in plant tissues and is localized to the ER, Golgi apparatus, prevacuolar compartment, and plasma membrane. The knockout mutants of svb showed enhanced tolerance to ER stress, which was genetically complemented by transducing genomic SVB. SVB showed time-dependent induction after tunicamycin-induced ER stress, which depended on IRE1 and bZIP60 but not bZIP17 and bZIP28 in the unfolded protein response (UPR). A protein–lipid overlay assay showed specific binding of SVB to phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. SVB is therefore suggested to be the plant-specific phosphoinositide-binding protein whose expression is controlled by the UPR through the IRE1-bZIP60 pathway in Arabidopsis.

In eukaryotic cells, the endoplasmic reticulum (ER) serves as a major site for secretory protein biosynthesis (Dobson, 2003). Nascent peptides that engage the secretory pathway first enter the ER lumen, where they are folded by the aid of molecular chaperones and enzymes catalyzing protein folding (Hartl and Hayer-Hartl, 2002). Properly folded proteins are allowed to translocate to the Golgi apparatus for further modification and targeting. However, under stressful conditions, newly synthesized proteins consume the folding capacity of the ER, causing aberrant proteins to accumulate in the ER. The accumulation of unfolded or misfolded proteins in the ER causes ER stress (Walter and Ron, 2011, 2013), which must be resolved for cell survival. To cope with ER stress, eukaryotic cells from yeasts to mammals and plants have evolved highly conserved signaling pathways to relieve the burden of unfolded proteins in the ER (Howell, 2013). These pathways are collectively termed the unfolded protein response (UPR).

The plant UPR consists of two signal transduction cascades (Howell, 2013). Inositol-requiring enzyme 1 (IRE1) is an ER-localized dual-functioning protein kinase and RNase (Noh et al., 2002) that splices bZIP60 mRNA to produce the active transcription factor bZIP60 under ER stress (Deng et al., 2011). bZIP60 protein enters the nucleus to upregulate the expression of UPR genes, including binding proteins BiP1 and BiP3, which serve as molecular chaperones in the ER to assist protein folding (Iwata et al., 2008). On the other hand, ER-localized membrane proteins bZIP17 and bZIP28 are translocated from the ER to the Golgi apparatus, where they are cleaved by the proteases site-1 protease (S1P) and S2P under ER stress (Liu et al., 2007b, 2007a; Iwata et al., 2017). The released protein fragments of bZIP17 and bZIP28 enter the nucleus to induce the expression of UPR genes, which enhance the protein-folding capacity. Hence, bZIP17 and bZIP28 function as transcription factors together with bZIP60...
under ER stress. Overall, ER protein homeostasis is maintained in the cells by the ER stress response.

The highly conserved UPR pathways transduce stress signals from the ER to the nucleus to elicit an ER stress response (Howell, 2013). Several unique UPR mediators were recently reported in plants; they act as a bridge to transduce cellular signals between the plasma membrane and nucleus. For example, a membrane-associated transcription factor, NAC062, undergoes proteolysis under ER-stressed conditions and relocates from the plasma membrane to the nucleus, followed by the transcriptional upregulation of UPR genes (Yang et al., 2014a). The β-subunit of the Arabidopsis (Arabidopsis thaliana) heterotrimeric G protein is another UPR transducer that functions in parallel with IRE1 and positively contributes to ER stress tolerance (Chen and Brandizzi, 2012; Cho et al., 2015). Furthermore, our previous study of Arabidopsis phosphoinositide-interacting proteins and microarray analyses. According to Oxley published data for phosphoinositide-signaling and the ER stress response, we searched membrane-localized proteins participate in the ER stress response (Kanehara et al., 2015). However, how plasma membrane-localized proteins participate in the ER stress response remains elusive.

To identify novel factors that link phosphoinositide signaling and the ER stress response, we searched published data for phosphoinositide-interacting proteins and microarray analyses. According to Oxley et al. (2013), 65 proteins are bound to phosphatidylinositol 3-phosphate (PI3P) or phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) during salinity stress in vitro (Oxley et al., 2013), and we searched whether those genes encoding the PI3P/PI(3,5)P2-binding proteins are transcriptionally regulated upon ER stress by using previously reported microarray data (Kamauchi et al., 2005; Iwata et al., 2010). One of the 65 genes, At1g56580, was induced under ER stress. At1g56580 was annotated as having unknown function and was designated as SMALLER TRICHOMES WITH VARIABLE BRANCHES (SVB) because of its mutant phenotype (Marks et al., 2009).

In this study, we showed that SVB is transcriptionally induced by tunicamycin (Tm)-induced ER stress and is required for ER stress tolerance. The upregulation depends on the IRE1-bZIP60 pathway but not on bZIP17 or bZIP28. SVB is localized to the ER, Golgi apparatus, prevacuolar compartment (PVC), and plasma membrane, and exhibits phosphoinositide-binding properties. We concluded that SVB is the plant-specific phosphoinositide-binding protein whose expression is controlled by the UPR for ER stress tolerance.

RESULTS

SVB Encodes a DUF538 Family Protein that Is Highly Conserved in Plants and Is Widely Expressed in Arabidopsis Tissues

The gene SVB (At1g56580) encodes a protein with 18-kD molecular mass that comprises 166 amino acids and contains one predicted domain of unknown function (DUF538) that ranges from amino acid K26 to P138 (Fig. 1A). Proteins that contain the DUF538 domain are termed the DUF538 family proteins. We first searched the public database PhylomeDB (http://phylomedb.org/; Huerta-Cepas et al., 2014) to identify SVB-like genes. SVB orthologs were found almost exclusively in Viridiplantae (green plants), including maize (Zea mays), rice (Oryza sativa), and Arabidopsis, but not in animals (Supplemental Fig. S1). The Arabidopsis genome contains six SVB homologs that are highly conserved in their DUF538 domains (Fig. 1B; Supplemental Fig. S2). We analyzed the primary sequence of the SVB protein by using the TargetP server (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2007) but found no signal sequence for the secretory pathway, chloroplast transit peptide, or mitochondrial targeting peptide. Furthermore, the tertiary structure of the SVB protein was analyzed by using the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2) in the intensive modeling mode (Kelley et al., 2015). SVB possessed multiple β-strands that are not likely membrane-spanning domains (Fig. 1C), so SVB may not be a membrane protein.

Next, to examine the expression profiles of SVB homologs in different developmental stages, we used GENEVESTIGATOR (http://www.genevestigator.com) to analyze public microarray data (Hruz et al., 2008). SVB was highly expressed in germinating seeds, seedlings, rosette leaves, young and mature flowers, and siliques but showed low expression in senescent leaves (Fig. 1D). At1g09310 showed a similar expression profile and level. By contrast, the expression of At4g24130, At5g46230, and At1g30020 was lower. At5g46230 was highly expressed in senescent leaves, whereas the others were not. Data for At5g49600 were not available in GENEVESTIGATOR software. Regarding tissue specificity, SVB and its homologs were expressed in most tissues with distinct tissue specificity (Fig. 1D). These data indicate that SVB encodes a DUF538 family protein that is highly conserved in green plants and is highly expressed at different developmental stages.

SVB Is Highly Expressed at Different Developmental Stages

To investigate the function of SVB, we examined the tissue-specific expression pattern by creating transgenic plants that expressed β-glucuronidase (GUS) reporter-tagged SVB driven by an endogenous promoter (ProSVB:SVB-GUS) in the svb-1 mutant. The svb-1 mutant is described in the following section. Twenty-four T1 plants were screened, and T2 lines 5, 10, and 16 were selected as representative lines (Fig. 2; Supplemental Fig. S3). GUS staining assay revealed that SVB was strongly expressed in vegetative tissues, including germinating seeds (Fig. 2A), young seedlings (Fig. 2B), cotyledons, hypocotyl and lateral root primordia (Fig. 2,
B and C), rosette leaves (Fig. 2D), and trichomes (Fig. 2E). In reproductive tissues, the SVB-GUS signal was found in the petals and pistils in young flowers (Fig. 2, F and G). In developed flowers, expression was observed in the stamens, pistils (Fig. 2, G and H), and siliques (Fig. 2I). Hence, SVB is expressed at different developmental stages, which is consistent with the gene expression pattern of SVB (Fig. 1D).

The svb Mutants Exhibit Aberrant Trichomes That Are Complemented by SVB Expression

To further investigate the function of SVB in Arabidopsis, we isolated two transfer DNA (T-DNA) knockout mutants of SVB: svb-1 (SALK_073071C) and svb-4 (GABI_053F05) by PCR-based genotyping (Fig. 3A). The positions of each T-DNA insertion were confirmed by sequencing. Both T-DNA insertions were located in the exon because SVB contains a sole exon with no introns (Fig. 3A). Reverse transcription quantitative PCR (RT-qPCR) analysis of SVB revealed that its expression in both svb-1 and svb-4 mutants was significantly lower than that in the wild type (Fig. 3B; 0.0127 ± 0.0004 in svb-1; 0.0528 ± 0.0015 in svb-4), which suggests that they are both null mutants.

A previous study reported that the svb mutant exhibits an aberrant trichome phenotype (Marks et al., 2009). Indeed, rosette leaves of 14-d-old svb-1 plants showed trichomes, with variable branch numbers, that were smaller than those of the wild type (Fig. 3C). We analyzed the phenotype by calculating the proportion of trichomes with different branch numbers on rosette leaves of 14-d-old plants. As shown in Table 1, 86% of wild-type trichomes developed three branches as compared with only 24% of trichomes of the svb-1 mutant. By contrast, a major portion of svb-1 trichomes developed one (12.9%) or two (61.2%) branches, whereas trichomes with these branching numbers were barely found in the wild type (one branch: 0.9%; two branches: 11.7%). To investigate whether the aberrant trichome phenotype was caused by knocking out SVB, we created transgenic plants that expressed SVB under its own promoter (ProSVB:SVB) in the svb-1 mutant because a previous study failed to complement the phenotype with the GLABRA2 (GL2) promoter (Marks et al., 2009). Two independent transgenic lines, svb-1 ProSVB:SVB lines 7 and 15, successfully rescued the
phenotype (Fig. 3C). The quantitative result of svb-1 genomic complementation lines also indicated the recovery of the aberrant trichome proportion (Table 1). Therefore, the impaired trichome development in the svb mutants may be due to the loss of SVB.

SVB Is Involved in ER Stress Tolerance and Is Transcriptionally Controlled by IRE1 and bZIP60

Previous microarray analyses showed that the expression of SVB was upregulated by treatment with ER-stress inducers, including Tm, dithiothreitol, and L-azetidine-2-carboxylic acid (Kamauchi et al., 2005; Iwata et al., 2010). However, whether SVB is indeed involved in ER stress tolerance remains unknown. Hence, we examined the Tm sensitivity of the svb-1 and svb-4 mutants by growing plants on one-half-strength Murashige and Skoog (MS) agar plates containing Tm. Tm has been commonly used to elicit ER stress by its inhibition of a biosynthetic pathway for N-linked protein glycosylation (Koizumi et al., 1999). We did not observe significant differences among the wild-type, svb-1, and svb-4 seedlings under the mock condition (Fig. 4A; Supplemental Fig. S4A). By contrast, both svb-1 and svb-4 seedlings showed enhanced tolerance on Tm-containing plates, as evidenced by larger sizes of rosette leaves (Fig. 4A; Supplemental Fig. S4A). Here, the ire1a-1 ire1b-1 double mutant served as a control for the Tm-hypersensitive phenotype (Chen and Brandizzi, 2012; Deng et al., 2013). Homozygous T-DNA–tagged mutants of ire1a-1 (SALK_018112C), ire1b-1 (GABI_638B07), and the double mutant of ire1a-1 ire1b-1 were previously reported (Deng et al., 2011; Nagashima et al., 2011; Nguyen et al., 2019). To quantify the result, we measured the fresh weight of
14-d-old seedlings. The fresh weight was significantly higher for both svb-1 and svb-4 mutants than the wild type under the Tm-treated condition (Fig. 4A; Supplemental Fig. S4A). We reasoned that knocking out SVB caused the Tm-tolerant phenotype in the svb mutants. Indeed, the two independent transgenic lines, svb-1 ProSVB:SVB lines 7 and 15, showed a complementary phenotype in the Tm-sensitivity assay (Fig. 4B; Supplemental Fig. S4B) because both lines rescued the trichome phenotype described in Table 1. To test whether overexpression of SVB alters ER stress tolerance, we produced transgenic lines that expressed SVB in wild-type plants. However, no significant effect was observed (Supplemental Fig. S5). These data indicate that SVB may contribute to ER stress tolerance.

To determine genetic interactions between SVB and UPR genes, we generated a triple mutant, svb-1 ire1a-1 ire1b-1, and performed epistasis analysis between SVB and the UPR sensor IRE1. We found no significant difference among the wild type, svb-1, ire1a-1 ire1b-1 double mutant, and svb-1 ire1a-1 ire1b-1 triple mutant under the mock condition (Fig. 5A). When plants were challenged with Tm, the svb-1 mutant showed enhanced tolerance, as described in Figure 4, whereas

Table 1. Proportion of trichomes with different branch numbers in wild type, svb-1 mutant, and svb-1 ProSVB:SVB transgenic lines 7 and 15

| Line                        | Number of Branches |
|-----------------------------|--------------------|
|                             | 1          | 2          | 3          | 4          |
| %                           |            |            |            |            |
| Wild type                   | 0.9 ± 1.4  | 11.7 ± 5.2 | 86.0 ± 6.5 | 1.5 ± 1.2  |
| svb-1                       | 12.9 ± 4.2***| 61.2 ± 8.1***| 24.5 ± 4.6***| 1.5 ± 1.6  |
| svb-1 ProSVB:SVB line 7     | 1.2 ± 1.3   | 23.9 ± 3.9**| 74.3 ± 4.6**| 0.6 ± 1.6  |
| svb-1 ProSVB:SVB line 15    | 0.0 ± 0.0   | 18.0 ± 8.7 | 80.4 ± 8.3 | 1.5 ± 1.2  |

Values indicate mean ± sd. Data were analyzed by Student’s t test with the corresponding wild-type data as the control. **P < 0.01 and ***P < 0.001.
ire1a-1 ire1b-1 and svb-1 ire1a-1 ire1b-1 were both Tm-hypersensitive (Fig. 5A). This result implies that ire1a-1 ire1b-1 is epistatic to svb-1.

To determine how transcriptional regulation of SVB expression was achieved, we performed RT-qPCR with 7-d-old seedlings of wild type and knockout mutants of UPR genes. The homozygous T-DNA–tagged mutant of bzip60-1 (SALK_050203C) was reported previously (Zhang et al., 2015; Nguyen et al., 2019). We isolated homozygous T-DNA–tagged mutants of bzip17-4 (GABI_220B01) and bzip28-3 (SALK_114900) by PCR-based genotyping and confirmed the positions of the T-DNA insertions by sequencing (Supplemental Fig. S6). Both lines were reported in Nagashima et al. (2011) and Kim et al. (2018). We found no significant difference in expression of SVB among the mutants of UPR genes at the zero-time point (mock condition; Fig. 5B). SVB was greatly induced after 5 h of Tm treatment in the wild type (Fig. 5B). The induction of SVB by Tm was completely abolished in the ire1a-1 ire1b-1 double mutant and the bzip60-1 mutant (Fig. 5B). By contrast, a similar induction pattern and level were found in the bzip17-4 and bzip28-3 mutants as compared with the wild type. These data suggest that SVB is transcriptionally controlled by IRE1 and bZIP60 but not bZIP17 and bZIP28 in the UPR under ER stress.

ER Stress Marker Genes Are Upregulated in the svb-1 Mutant during the Acute ER Stress Response

During Tm-induced ER stress, activation of UPR pathways leads to an induction of multiple ER stress-responsive marker genes, including Bip3, calnexin 1 (CNX1), and
calreticulin 1 (CRT1; Kamauchi et al., 2005; Cho et al., 2015).

To investigate whether SVB is involved in controlling these ER stress-responsive genes, we treated 7-d-old wild-type and svb-1 seedlings with Tm for 0 (mock condition), 2, and 5 h and analyzed gene expression by RT-qPCR. Under these conditions, all the genes tested here were upregulated in both the wild type and svb-1 mutant, while the expression of BiP3 and CNX1 was higher in svb-1 than in the wild type (Fig. 6, A–C). The expression of BiP3 (Fig. 6D) and CRT1 (Fig. 6F) was higher in svb-1 than in the wild type at the zero-time point (mock condition). These results suggest that SVB is involved in controlling the expression of several UPR genes during the ER stress response.

SVB Binds to PI(3,5)P2 and PI(3,4,5)P3 In Vitro

SVB is one of the PI3P- and PI(3,5)P2-binding proteins in Arabidopsis (Oxley et al., 2013). However, whether SVB also binds other phosphoinositide species is not known. To explore the phosphoinositide-binding specificity of SVB protein, we performed the protein–lipid overlay assay described in Nakamura et al. (2014). SVB was first fused to the C terminus of maltose binding protein (MBP), then expressed in Escherichia coli C41(DE3) cells (Fig. 7A). Subsequently, MBP-SVB protein was purified by using amylose resin and hybridized with membrane-dotted phosphoinositides, including PI3P, PI4P, PI5P, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3 (Fig. 7B). The bindings were detected by using anti-MBP antibody. When we performed this assay with enriched proteins (2 μg), strong binding of MBP-SVB to PI(3,5)P2 and PI(3,4,5)P3 was detected, whereas binding to PI4P and PI5P was comparably weaker (Fig. 7C). We did not detect the specific binding of MBP-SVB to PI3P under our condition, although a previous study reported this binding (Oxley et al., 2013). When we decreased the MBP-SVB protein amount to one-fourth during lipid overlay, it still bound to PI(3,5)P2 and PI(3,4,5)P3 with no clear
binding detected toward the other PIs (Fig. 7C). The purified MBP protein itself was used as a negative control and no specific binding was detected (Fig. 7C). Our results suggest that SVB binds several phosphoinositide species specifically, including PI(3,5)P2 and PI(3,4,5)P3.

**SVB Is Localized to the ER, Golgi Apparatus, PVC, and Plasma Membrane**

Our results from the Tm-sensitivity assay, RT-qPCR analysis, and protein–lipid overlay assay suggest that SVB may be a phosphoinositide-binding protein that mediates the ER stress response. To better understand the intracellular function of SVB, we created transgenic plants that express genomic SVB tagged with the fluorescent protein Venus (ProSVB:SVB-Ven) in svb-1 plants to study the subcellular localization of SVB. Twenty-four T1 transgenic lines were screened, and T2 lines 9 and 18 were used as representative lines for observation. These transgenic lines rescued the aberrant trichome phenotype (Table 2). We first observed the lateral roots of 7-d-old svb-1 ProSVB:SVB-Ven seedlings because we observed high expression of SVB in this tissue in the GUS-reporter assay (Fig. 2). A major portion of the SVB-Ven signal colocalized with the ER-specific dye ER Tracker Red (Fig. 8A), and a minor portion merged with the plasma membrane dye FM4-64 (Fig. 8B). These data indicate that SVB may be

Figure 6. ER stress-responsive genes show increased expression in the svb-1 mutant. A to C, RT-qPCR analysis of the expression of the ER stress-responsive genes BiP3 (A), CNX1 (B), and CRT1 (C) in 7-d-old wild-type (WT) and svb-1 plants in response to ER stress. Plants were grown on one-half-strength MS agar plates for 7 d, then transferred to one-half-strength MS liquid medium containing 5 μg mL⁻¹ Tm for the times indicated. For the zero-time point, plants were treated with DMSO for 5 h. The expression in the wild type at the zero-time point was set to 1, and relative expression is shown. Three biologically independent experiments were performed with similar results. Data are mean ± SD from one representative experiment with three technical replicates. Different letters indicate significant difference among genotypes at *P < 0.05, as determined by one-way ANOVA. D to F, Expression of BiP3 (D), CNX1 (E), and CRT1 (F) at the zero-time point in the wild type and svb-1 as shown in A, B, and C, respectively. Student’s t test was used for statistical analysis. Asterisks indicate significance between wild type and svb-1 (**P < 0.01 and ***P < 0.001).
localized to the ER and the plasma membrane in the lateral root. We also found several intracellular SVB-Ven puncta that did not perfectly merge with the ER Tracker Red or FM4-64 signal (Fig. 8, A and B, white arrowheads). This result prompted us to investigate whether these fluorescent puncta represent intracellular vesicles. To further examine the subcellular localization of SVB, we transiently expressed SVB-Ven under the native promoter (ProSVB:SVB-Ven) together with 35S promoter-driven AtWAK2 (signal peptide)-mCherry-HDEL (ER-rk) or GmMan (1-49)-mCherry (G-rb) in the leaf epidermal cells of Nicotiana benthamiana. ER-rk and G-rb were previously reported as the ER and Golgi markers in colocalization studies, respectively (Nelson et al., 2007). Consistent with the observations in our stable transgenic lines, SVB-Ven colocalized well with the ER marker ER-rk (Fig. 8C). Additionally, SVB-Ven colocalized with the Golgi marker G-rb in the merged image (Fig. 8D). Detailed examination of Z-stack serial images confirmed the colocalization of SVB-Ven with G-rb in different focal planes (Supplemental Fig. S7). Furthermore, SVB was verified as a PI(3,5)P₂-binding protein in this study, and PI(3,5)P₂ is known to be exclusively localized on the late endosome/PVC (Hirano et al., 2017). Therefore, we tested whether SVB-Ven could merge with a previously reported PVC marker, mRFP-RabF2b (Kotzer et al., 2004). SVB-Ven was found in contact with mRFP-RabF2b in both the merged image and Z-stack series (Fig. 8E; Supplemental Fig. S8). Notably, perfect colocalization was observed in intracellular vesicles in several Z focal planes (Supplemental Fig. S8). Similar colocalization patterns were also observed in svb-1 ProSVB:SVB-Ven transgenic plants transiently expressed with ER-rk, G-rb, mRFP-RabF2b, and a previously reported plasma membrane marker, pm-rb (Nelson et al., 2007; Supplemental Fig. S9). Overall, these data suggest that SVB is mainly localized to the ER and may also localize to the Golgi apparatus, PVC, and plasma membrane.

DISCUSSION

In our previous study of AtPLC2 (Kanehara et al., 2015), we demonstrated the involvement of phosphoinositide signaling in the ER stress response. To investigate the molecular mechanisms linking both cellular processes, we searched the literature for phosphoinositide-binding proteins and microarray analyses for ER stress (Kamauchi et al., 2005; Iwata et al., 2010; Oxley et al., 2013). Here, we report the identification of a phosphoinositide-binding protein, namely SVB, as a plant-specific mediator linking phosphoinositides and UPR based on the following...
SVB encodes a DUF538 family protein that is highly conserved in plants and is widely expressed in tissues of Arabidopsis; (2) the knockout mutants svb-1 and svb-4 showed enhanced tolerance to Tm-induced ER stress as compared with wild-type plants; (3) the expression of SVB was transcriptionally upregulated during Tm-induced ER stress, and this regulation depended on IRE1 and bZIP60 but not bZIP17 or bZIP28; (4) the ER stress-responsive genes showed higher expression in the svb-1 mutant than wild-type plants; (5) the protein–lipid overlay assay showed that SVB interacts with multiple phosphoconitidase species, including Pl(3,5)P2 and Pl(3,4,5) P3; and (6) the subcellular localization study in transgenic Arabidopsis plants and leaves of N. benthamiana revealed that the SVB protein is localized to the ER, Golgi apparatus, PVC, and plasma membrane.

Among the UPR, the IRE1-bZIP60 pathway is the most highly conserved pathway in yeast (designated as “Ire1-Hac1”), animals (designated as “IRE1-XBP1”), and plants, whereas the bZIP17/bZIP28 pathway is present in animals (designated as “ATF6”) and plants but not in yeast. The third pathway of the UPR in animals is mediated by a double-strand RNA-activated protein kinase-like ER kinase (PERK), which phosphorylates eIF2α to attenuate mRNA translation and, in turn, reduces the newly synthesized protein load in the ER (Walter and Ron, 2011). However, whether the PERK pathway exists in plants remains questionable because no functional PERK orthologs have been identified in plants yet. In this study of SVB, we found that SVB belongs to the DUF538 protein family that contains a conserved domain (DUF538) of unknown function (Fig. 1, A and B). Based on the data from the UniProt Knowledgebase (https://www.uniprot.org/), the Arabidopsis genome encodes 60 DUF538 family proteins. Members of this protein family have been frequently found as stress-induced proteins in plants (Gholizadeh, 2011; Gholizadeh and Kohnehrouz, 2013; Takahashi et al., 2013). Among these members, a plant DUF538 protein, At5g37070, was predicted as a homolog of mammalian bactericidal permeability increasing protein that plays roles in immunity against different pathogens (Gholizadeh and Kohnehrouz, 2013). A photoconvertible chlorophyll binding protein (CaWSCP) identified in Chenopodium album was reported as a member of the DUF538 family (Takahashi et al., 2013). According to the UniProt Knowledgebase (https://www.uniprot.org/) and data from the Phylome database (Fig. 1B; Supplemental Fig. S1), SVB orthologs were found almost exclusively in Viridiplantae. Thus, SVB is most likely one of the plant-specific proteins related to various stress responses.

SVB was first identified as a gene required for trichome development based on the transcriptomic analysis of the Arabidopsis gl3-sst sim mutant (Marks et al., 2009). Consistently, we observed similar aberrant trichomes in the svb mutants that exhibited a variable branching number (Fig. 3C; Table 1). In the previous study, the complementation assays failed to rescue the aberrant trichome phenotype by expressing GLABRA2 promoter-driven SVB or SVB fused to GFP (Marks et al., 2009). Here, we successfully rescued the phenotype by expressing SVB under its own promoter (Fig. 3C; Tables 1 and 2), which indicates that SVB is indeed required for trichome development. Moreover, our GUS-reporter assay in Arabidopsis transgenic plants revealed that the SVB protein is highly expressed in the trichomes of rosette leaves (Fig. 2E; Supplemental Fig. S3, I and J), a finding that supports the cellular function of SVB in plant development. Overall, we provide further evidence for the role of SVB in trichome development.

Our Tm-sensitivity assay suggested that SVB is involved in the ER stress response because both svb-1 and svb-4 mutants showed enhanced tolerance to Tm-induced ER stress as compared with the wild type (Fig. 4A; Supplemental Fig. S4A). The phenotype was complemented by expressing genomic SVB (Fig. 4B; Supplemental Fig. S4B), which suggests that the proper function of SVB is important for trichome development and also for ER stress tolerance. Previously, Arabidopsis WRKY15 was reported as a transcription factor involved in osmotic stress tolerance that may work together with WRKY8 to regulate trichome patterning (Jakoby et al., 2008; Vanderauwera et al., 2012). The genes involved in the ER stress response and the UPR were significantly enriched in WRKY15-overexpressed plants (Vanderauwera et al., 2012), which may provide a possible link to address how SVB functions to regulate both trichome development and ER stress tolerance.

The expression of SVB was induced in wild-type plants during the time-course treatment of Tm (Fig. 5B), a finding that is consistent with published microarray data (Kamauchi et al., 2005; Iwata et al., 2010). This upregulation of SVB was abolished in both

| Line          | Number of Branches | % |
|---------------|--------------------|---|
| Wild type     | 1.4 ± 2.5          | 26.3 ± 12.8 | 71.3 ± 12.9 | 1.0 ± 1.5 |
| svb-1         | 34.7 ± 5.4***      | 49.9 ± 10.8** | 14.2 ± 8.0*** | 1.1 ± 1.2 |
| svb-1 ProSVB:SVB-Ven line 9 | 8.2 ± 10.2 | 36.5 ± 22.7 | 54.2 ± 30.1 | 0.8 ± 1.9 |
| svb-1 ProSVB:SVB-Ven line 18 | 11.7 ± 7.9* | 43.5 ± 9.7* | 44.1 ± 8.4** | 0.6 ± 1.5 |
ire1a-1 ire1b-1 and bzip60-1 mutants but not in bzip17-4 or bzip28-3 mutants (Fig. 5B), which suggests that one SVB function may be as a downstream mediator of the IRE1-bZIP60 branch in the UPR. Furthermore, our genetic epistasis analysis supported the idea that IRE1 and SVB play a role in the same branch of UPR, in part because the ire1a-1 ire1b-1 double mutant is epistatic to the svb-1 mutant (Fig. 5A).

Our protein–lipid overlay assay demonstrated that SVB specifically binds to PI(3,5)P₂ and PI(3,4,5)P₃ (Fig. 7C). In addition to the previously reported interaction between SVB and PI(3,5)P₂ (Oxley et al., 2013), PI(3,4,5)P₃ was newly identified as the binding partner in our study. Among all plant phosphoinositide species, the existence of PI(3,4,5)P₃ is still debated in plants (Heilmann, 2016). In contrast to a previous report (Oxley et al., 2013), we did not detect an interaction between SVB and PI3P under our conditions (Fig. 7C). This discrepancy may be due to the difference in sample preparation and experimental methodologies. In conclusion, the two independent studies indicate that PI(3,5)P₂ may be a binding partner of SVB.

Figure 8. SVB is localized to the ER, Golgi apparatus, PVC, and plasma membrane. A and B, Colocalization studies of SVB-Ven in lateral roots of 7-d-old svb-1 ProSVB:SVB-Ven transgenic plants (line 9). A, The signals of SVB-Ven, ER Tracker Red, differential interference contrast (DIC), and merged images are presented from left to right. White arrowheads indicate SVB-Ven puncta. B, The signals of SVB-Ven, FM4-64, DIC, and merged images are presented from left to right. White arrowheads indicate SVB-Ven puncta. C to E, Transient expression of SVB-Ven and organelle markers in N. benthamiana leaf epidermal cells. C, The signals of SVB-Ven, ER-rk, and merged images are presented from left to right. D, The signals of SVB-Ven, G-rb, and merged images are presented from left to right. E, The signals of SVB-Ven, mRFP-RabF2b, and merged images are presented from left to right. Scale bars = 5 μm (A–C) and 20 μm (D and E).
Our subcellular localization study revealed that SVB is localized to multiple cellular compartments, including the ER, Golgi apparatus, PVC, and plasma membrane (Fig. 8; Supplemental Figs. S7, S8, and S9). These organelles present phosphoinositides as membrane lipids, which are important for their physiological function (Gether et al., 2017). For example, AtSAC1 (suppressor of actin 1) is a PI(3,5)P2 phosphatase associated with the Golgi apparatus, which regulates the secretory pathway and is crucial for cell wall deposition (Zhong et al., 2005). Phosphatidylinositol 3-phosphate 5-kinase FAB1, which produces PI(3,5)P2, is predominantly located in PVC/late endosomes and regulates endosome maturation (Hirano et al., 2015). The disruption of PI(4,5)P2 homeostasis by manipulating PIP5K6 (suppressor of actin 1) is a PI(3,5)P2 phosphatase asso-
dicated by impaired endocytosis at the tip of the pollen tube (Zhao et al., 2010). Regarding the expression of the SVB gene achieves better growth during ER stress. (2) SVB is a soluble cytoplasmic protein based on its amino acid sequence. However, our observation clearly demonstrated that SVB exists in cellular compartments in the secretory pathway, including the ER, Golgi apparatus, PVC, and plasma membrane. SVB may require binding partners that localize in the secretory pathway, such as phosphoinositide species and unknown proteins, for its proper localization.

This study of SVB provides additional support for the hypothesis that phosphoinositide signaling is involved in the plant ER stress response. Because the svb mutants showed enhanced tolerance to ER stress as shown in Figure 4, it is important to address how the mutation in SVB gene achieves better growth during ER stress. Persistent ER stress is known to lead to cell death, which is controlled by the IREs in mammals and plants (Tabas and Ron, 2011; Mishiba et al., 2013). In Arabidopsis, the ER membrane-associated transcription factor NAC089 promotes programmed cell death under ER stress (Yang et al., 2014a, 2014b). The following three features are described for NAC089 (Yang et al., 2014b): (1) the knock-down mutants of NAC089 are more toler-
antly to ER stress; (2) NAC089 is upregulated upon ER stress through bZIP60 and bZIP28; and (3) NAC089 is substantially expressed under normal conditions. Because our data demonstrate features similar to NAC089 regarding the above-mentioned three points, SVB may contribute to programmed cell death under ER stress through the IRE1-bZIP60-NAC089 pathway. Notably, whether upregulation of SVB gene induced by Tm is necessary for ER stress tolerance is unclear because the expression of the SVB gene is high under nonstressed conditions (Figs. 1D and 2). The spatiotemporal dynamics of phosphoinositides in different cellular compartments is well-established to be crucial for their function (Gether et al., 2017). Upregulation of SVB may be required to meet a demand for enriched phosphoinositides at some subcellular location under ER stress. In addition, our subcellular localization study of SVB-Ven showed that SVB was localized to the ER, Golgi apparatus, PVC, and plasma membrane in Arabidopsis transgenic plants (Fig. 8; Supplemental Figs. S7, S8, and S9). These subcellular localizations are not fully explained by binding only to PI(3,5)P2 because PI(3,5)P2 is known to be enriched in PVC (Hirano et al., 2017) but not the ER, Golgi apparatus, or plasma membrane. SVB may have some other binding partners other than PI(3,5)P2 in vivo.

In conclusion, SVB may be the plant-specific phosphoinositide-binding protein that mediates the UPR through the IRE1-bZIP60 pathway in Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) plants (Columbia-0 ecotype) were used in this study and were grown under a continuous light condition at 22°C. T-DNA-tagged mutant lines for sbi-1 (SALK_030717C), ire1a-1 (SALK_018112C), bzip60-1 (SALK_050203C), and bzip28-3 (SALK_114900) were obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). T-DNA-tagged mutant lines for sbf-4 (GABI_053F05), irelb-1 (GABI_638B07), and bzip17-4 (GABI_22B01) were obtained from the European Arabidopsis Stock Centre (https://www.gabi-kat.de). Seeds acquired from stock centers were surface-sterilized with 70% (v/v) ethanol, then germinated on one-half-strength Murashige and Skoog (MS) agar plates (Murashige and Skoog, 1962). The double and triple mutants were created by genetic crossing of the respective single mutant. Homozygous mutants were identified by PCR-based genotyping (O’Malley et al., 2015) with the primers sbf-1 (LB: KK8; LP: CY40; RP: CY41), sbi-4 (LB: YN1016; LP: CY52; RP: CY53), ire1a-1 (LB: KK5; LP: KK525; RP: KK526), ire1b-1 (LB: YN1016; LP: KK528; RP: KK527), bzip60-1 (LB: KK8; LP: KK536; RP: KK535), bzip17-4 (LB: YN1016; LP: KK537; RP: KK538), and bzip28-3 (LB: KK8; LP: KK534; RP: KK533). The locations of the T-DNA insertion sites of bzip17-4 and bzip28-3 were confirmed by DNA sequencing (Supplemental Fig. S6). The mutants of ire1a-1, ire1b-1, irela-1relb-1, and bzip60-1 were described in Deng et al. (2011), Nagashima et al. (2011), and Nguyen et al. (2019). The nucleotide sequences of the primers are in Supplemental Table S1.

For the transient expression study of SVB-Ven, Nicotiana benthamiana plants were grown under a continuous light condition at 22°C for 4 to 5 weeks. Leaves with adequate size on plants before bolting were excised for particle bombardment as described below.

Quantification of the Trichome Phenotype

Trichomes on the adaxial surface of the third or fourth rosette leaves on 14-d-old plants were observed (~350 trichomes counted) and classified as trichomes with one, two, three, and four branches, respectively. Six leaves on six independent plants per genotype were used. Data were analyzed by Student’s t test, with the corresponding wild-type data as the control.

Plasmid Construction and Plant Transformation

For complementation of the sbi-1 mutant, the genomic SVB (ProSbVSb:SVB) sequence that includes a 1-kb fragment with the 5′-untranslated region (UTR), coding region, and 0.5-kb 3′-UTR was amplified with the primers CY162 and CY147 and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). Arabidopsis wild-type genomic DNA was used as a template. The amplified fragment was cloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific) to obtain pCY73. Thereafter, pCY73 was recombined with the pBGW destination vector (Nakagawa et al., 2009) by using Gateway LR Clonase II.
(Thermo Fisher Scientific). The obtained pCY74 (pBGW-ProSVB:SVB) was transduced into nbs-1 plants by Agrobacterium-mediated transformation. Transformants were selected by spraying 0.1% (v/v) BASTA (Bayer Crop Science) solution on soil-growing seedlings. In total, 16 independent transgenic plants were screened. Lines 7 and 15 were selected for subsequent analyses.

To study the tissue expression pattern of SVB, pCY73 underwent site-directed mutagenesis with the CY163 primer to introduce the 5′-f0l site before the stop codon of SVB to obtain pCY76. Thereafter, a GUS reporter that encodes β-glucuronidase was cleaved out from the previously reported pYN2036 plasmid (Kanehara et al., 2015) by f0l (New England BioLabs) and inserted into pCY76 to obtain pCY77. Next, pCY77 was recombined with pBGW via Gateway LR Clonase II (Thermo Fisher Scientific). The obtained pCY114 (pBGW-ProSVB:SVB-GUS) was transduced into nbs-1 plants by Agrobacterium-mediated transformation. Transformants were selected by spraying 0.1% (v/v) BASTA (Bayer Crop Science) solution on soil-growing seedlings. In total, 24 independent transgenic plants were screened by genotyping and histological GUS staining. Lines 5, 10, and 16 were selected as representatives for observation.

To investigate the subcellular localization of SVB, a triple Venus (Ven) fluorescent tag was cleaved out from the previously reported pYN2037 plasmid (Kanehara et al., 2015) by f0l and inserted into pCY76 to obtain pCY80. Next, pCY80 was recombined with pBGW via Gateway LR Clonase II. The obtained pCY115 (pBGW-ProSVB:SVB-Ven) was transduced into nbs-1 plants by Agrobacterium-mediated transformation. Transformants were selected by spraying 0.1% (v/v) BASTA (Bayer Crop Science) solution on soil-growing seedlings. In total, 24 independent transgenic lines were screened by genotyping and observation of the Venus signal. Lines 6, 9, and 16 were selected as representatives for the colocalization study.

To create the PVC marker mRFP-Rab2b, the coding sequence of ArRab2b was amplified with primers CY308 and CY324, then cloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific) to obtain pCY104. Thereafter, pCY140 was recombined with the pGW6455 destination vector (Nakagawa et al., 2009) via Gateway LR Clonase II. The obtained plasmid pCY141 was used for the transient expression study with N. benthamiana.

To study the phosphoinositide-binding specificity of SVB, a pMAL protein fusion and purification system (New England BioLabs) was used. The coding sequence of SVB was amplified with Arabidopsis wild-type cDNA as a template with primers K746 and KK747, and the SVB fragment was cloned into the pMAL vector between the Ndel and BamHI sites. The obtained pCY35 was transformed into the Escherichia coli strain C41(DE3) for a recombinant protein expression. The nucleotide sequences of the primers are in Supplemental Table S1. All the site-directed mutagenesis experiments were performed as reported in Sawano and Miyawaki (2000).

**RNA Extraction and RT-qPCR**

Total RNA was extracted from seven seedlings of 7-d-old plants in each condition by using a RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNAs were synthesized by using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). RT-qPCR was performed with a model no. 7500 Real-Time PCR System (Applied Biosystems). The comparative Ct method was used to determine the relative amount of the target genes, with ACTIN2 expression as an internal control. At least three biologically independent experiments were performed with three technical replicates. The oligonucleotide primers for RT-qPCR are in Supplemental Table S1.

**Histological GUS Staining**

Plant tissues at various developmental stages were harvested and immersed in ice-cold 90% (v/v) acetone for 15 min, then samples were washed with 0.1 M sodium phosphate buffer (0.1 M NaH₂PO₄ and 0.1 M Na₂HPO₄) and incubated with GUS staining solution (10 mM EDTA, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.1% (v/v) Triton X-100, and 0.5 mg mL⁻¹ X-Glu) at 37°C in the dark for 16 h. The reaction was terminated by replacing GUS staining solution with 70% (v/v) ethanol. Color pigments were removed by immersing the tissue in ethanol-acetic acid = 6:1 (v/v) at room temperature.

**Tm Treatment**

For phenotypic observation, two methods were used: (1) plants were grown on one-half-strength MS agar plates for 4 d and transferred to one-half-strength MS agar plates containing 25 ng mL⁻¹ Tm dissolved in DMSO or just DMSO for an additional 10 d of culture and (2) plants were grown on one-half-strength MS agar plates containing 25 ng mL⁻¹ Tm or DMSO for 14 d of culture. Detailed phenotypic analysis was performed by measuring fresh weight and displayed as box-whisker plots. Each box represents the quartile above and below the median value. Whiskers represent the minimum and maximum values. For RT-qPCR analysis, plants were grown on one-half-strength MS liquid medium containing 5 μg mL⁻¹ Tm or DMSO. The seedlings were harvested at the times indicated. In the time-course experiments, plants for the zero-time point were treated with DMSO for 5 h.

**Protein–Lipid Overlay Assay**

MBP-fused recombinant proteins were purified according to the manual of the pMAL Protein Fusion and Purification System (New England BioLabs, 2005). Briefly, E. coli C41(DE3) cells harboring pCY53 (MBP-SVB) or the pMAL-c5X vector control (MBP) were grown in Terrific Broth medium containing ampicillin (100 μg mL⁻¹) and chloramphenicol (25 μg mL⁻¹) at 37°C. The expression of recombinant proteins was induced by adding 0.2 mM isopropyl β-D-thiogalactopyranoside for 2 h. The expressed proteins were purified with amylose resin (New England BioLabs), then quantified by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). For the protein–lipid overlay assay, purified proteins of various amounts (2 or 0.5 μg) were incubated with Amersham Hybond P membranes (GE Healthcare) that were dotted with 5 μg of phosphoinositide lipid species as indicated. The hybridization procedure was performed as described in Nakamura et al. (2014) except for the replacement of the primary antibody with an anti-MBP monoclonal antibody number E80325 (New England BioLabs). The recombinant proteins bound to lipid spots were detected by using the SuperSignal West Pico chemiluminescence substrate (Thermo Fisher Scientific) and were imaged by using an ImageQuant LAS4000 Imager (GE Healthcare). The phosphoinositide species used in this assay were purchased from Avanti Polar Lipids (Avanti) and are as follows: di18:1 PI(3,5)P₂ (Cat. no. 850152P), di18:1 PI(4,5)P₂ (Cat. no. 850154P), and di18:1 PI(3,4,5)P₃ (Cat. no. 850156P).

**Confocal Laser-Scanning Microscopy**

For the subcellular localization study of SVB-Ven, 7-d-old seedlings of ProSVB:SVB-Ven nbs-1 transgenic lines were observed using a model no. LSM510 Meta confocal microscope (Zeiss) equipped with a C-Apochromat 40×/1.20 W Korr UV-VIS-IR objective lens (Zeiss). Images were captured by using LSM510 v3.2 software and are analyzed by using ZEN software (both by Zeiss). For plasma membrane staining, 7-d-old seedlings were immersed in 5 μg mL⁻¹ FM4-64 (Invitrogen) for 5–5 s, washed with sterile water, then used for observation. For the ER staining, 7-d-old seedlings were stained with 1 μM ER-Tracker Red dye (Invitrogen) for 30 min, washed with sterile water, then used for observation.

**Transient Expression of SVB-Ven**

To transiently coexpress ProSVB:SVB-Ven (pCY115) with organelle markers ER-rk, G-rb, or mRFP-Rab2b (pCY141), plasmid DNAs at a 1:1 ratio were coated on leaves of N. benthamiana by using a Bio-Rad PDS-1000/He system. The particles were prepared by mixing 5 μg DNA, 2.5 mM CaCl₂, and 0.1 M spermidine with 1 mg tungsten microcarriers. The coated particles were pelleted, washed with absolute ethanol, and applied on macrocarriers (InBio Gold). The bombardment procedure was performed as described in the Bio-Rad instruction manual. The bombarded samples were recovered under continuous light for 48 h, then observed under an LSM880 confocal microscope equipped with an Airyscan module (Zeiss). Z-stack images were captured by using a C-Apochromat 40×/1.20 W Korr UV-VIS-IR objective lens and analyzed by using ZEN software (Carl Zeiss).

**Phylogenetic Analysis**

To identify SVB orthologs, gene locus ID of Arabidopsis SVB (Atg56580) was searched using the PhylomeDB v4 database (http://phylomedb.org/). Data represent the Phyl00012E2 free in Phylome 28 is shown as Supplemental

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Figure S1; data representing the Phy0001E2F tree in Phylome S02 is shown as Figure 1B.

Statistical Analysis

RStudio v1.2.1335 (https://www.rstudio.com/) was used for analysis by one-way ANOVA. Data were obtained from at least three biologically independent experiments. Significantly different groups among the conditions were further evaluated by using Tukey’s Honestly Significant Difference test. Means with different letters indicate significant difference. P < 0.05 was considered statistically significant. For Student’s t test, data were obtained from at least three biologically independent experiments and are displayed with asterisks to indicate differences among groups (’P < 0.05; ’’P < 0.01; ’’’P < 0.001; and ’’’’P < 0.0001).

Accession Numbers

Sequence data from this article can be found under the following Arabidopsis Genome Initiative accession number SVB (AtJG56380).

Supplemental Data

The following materials are available.

Supplemental Figure S1. Phylogenetic tree of SVB.

Supplemental Figure S2. Multiple sequence alignment of amino acids in SVB homologs.

Supplemental Figure S3. Tissue-specific expression of SVB by histochemical GUS staining of svb-1 ProSVB::SVB-GUS transgenic lines 10 and 16.

Supplemental Figure S4. SVB is involved in ER stress tolerance.

Supplemental Figure S5. Overexpression of SVB has no effect on ER stress tolerance.

Supplemental Figure S6. Isolation of izip17-4 (GABI_220B01) and izip28-3 (SALK_114900) mutants.

Supplemental Figure S7. SVB was localized to the Golgi apparatus.

Supplemental Figure S8. SVB was localized to the PVC.

Supplemental Figure S9. SVB was located to the ER, Golgi apparatus, PVC, and plasma membrane.

Supplemental Table S1. Oligonucleotide primers used in this study.

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