

Characterization of Cell Death Induced by *NbBPS1* Silencing in *Nicotiana benthamiana*

Yong Won Kang¹, Young Jeon¹, and Hyun-Sook Pai*

We previously showed that silencing of *NbBPS1* encoding an endoplasmic reticulum (ER)-localized protein results in pleiotrophic developmental defects and cell death in *Nicotiana benthamiana* [Kang et al. (2008)]. In this study, we investigated the mechanism of the cell death caused by *NbBPS1* silencing. Affected leaf cells exhibited morphological markers of programmed cell death (PCD) and accumulated excessive amounts of reactive oxygen species. *NbBPS1* silencing caused dramatic induction of the ER stress marker genes *BiP-like protein (BLP)* genes, *HSP70*, and *Bax Inhibitor-1*. Furthermore, *NbBPS1* deficiency led to relocalization of bZIP28 transcription factor from the ER membrane to the nucleus, similar to the bZIP28 relocalization during tunicamycin-induced ER stress. Abnormal accumulation of vesicles and increased autophagy activity were also observed in the affected leaf cells. These results suggest that inactivation of *NbBPS1* function in the ER leads to ER stress, autophagy, and PCD activation in *N. benthamiana*.

INTRODUCTION

Programmed cell death (PCD) is an active process associated with distinctive morphological and biochemical features. The process is essential for development and survival of multicellular organisms (Vaux and Korsmeyer, 1999; Wertz and Hanley, 1996). In plants, PCD is integral in development of xylem tracheary elements, leaf senescence, and the hypersensitive response to pathogen attack (Coll et al., 2011; Lam et al., 2001; Reape and McCabe, 2008). Environmental stresses such as nutrient deficiency, high salt, and drought also induce PCD in plants, suggesting that the process may confer resistance to stress. Recent studies have reported that endoplasmic reticulum (ER)-mediated stress can induce apoptotic pathways in animals and plants and that the ER stress-induced PCD is involved in plant responses to biotic and abiotic stresses (Costa et al., 2008; Szegezdi et al., 2006; Watanabe and Lam, 2006; 2008).

Protein folding in the ER is highly regulated under quality control surveillance. Nonetheless, inadequate nutrition, pathogen infection, hypoxia, changes in ER luminal Ca⁺⁺ stores, toxic chemicals, and mutations disrupt protein folding reactions in the

ER, leading to accumulation of unfolded proteins and protein aggregates. These conditions are detrimental to cell survival, and are called ER stress. To counteract ER stress, the cell activates the unfolded protein response (UPR), ER-associated degradation, and Ca⁺⁺ signaling (Boyce and Yuan, 2006; Høyer-Hansen and Jäättelä, 2007; Kaufman et al., 2002; Schröder and Kaufman, 2005). These pathways eventually reduce the ER load of unfolded proteins by decreasing global translation, increasing protein folding capacity in the ER, and stimulating degradation of misfolded proteins by the 26S proteasome or autophagy.

When ER stress is excessive or sustained, these signaling pathways can activate apoptosis in mammals (Li et al., 2006; Shiraishi et al., 2006; Szegezdi et al., 2006). The ER stress also activates autophagy, which functions to mitigate ER stress by removing aggregates and suppressing cell death, although excessive autophagy may also promote cell death (Ding and Yin, 2008; Høyer-Hansen and Jäättelä, 2007; Sakaki and Kaufman, 2008). In plants, tunicamycin (an inhibitor of N-linked protein glycosylation), cyclopiazonic acid (an inhibitor of ER calcium pumps), and water stress in roots elicit the ER stress and PCD (Crosti et al., 2001; Duan et al., 2010; Watanabe and Lam, 2008; Zuppini et al., 2004), but the mechanisms of the UPR pathway and the ER stress-induced PCD and autophagy are not well understood in plants.

Arabidopsis *BYPASS1 (BPS1)* encodes a protein with no homology to any characterized proteins (Van Norman et al., 2004). The *bypass1 (bps1)* mutant was originally identified by a screen for altered leaf vein patterns, and the mutant exhibited severe defects in shoot and root growth (Van Norman et al., 2004). In a previous study, we examined the function of *Nicotiana benthamiana* *BPS1 (NbBPS1)* in plant growth and development by studying the cellular effects of *NbBPS1* depletion in *N. benthamiana* (Kang et al., 2008b). The *NbBPS1:GFP* fusion protein was mainly associated with the endoplasmic reticulum. Virus-induced gene silencing (VIGS) of *NbBPS1* resulted in growth retardation, abnormal leaf development, and cell death (Kang et al., 2008b). The VIGS plants exhibited hyperproliferation of the cambial cells in the stem and elevated endoreduplication in the leaves. Cell death in *NbBPS1* VIGS leaves involved vacuole collapse followed by degeneration of the organelles (Kang et al., 2008b). These results suggest an essential function of *NbBPS1* in plant growth and development. In this

Department of Systems Biology, Yonsei University, Seoul 120-749, Korea, ¹These authors contributed equally to this work.
*Correspondence: hspai@yonsei.ac.kr

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study, we investigated the mechanism of cell death induced by *NbBPS1*-silencing in *N. benthamiana*. From our results, we suggest that *NbBPS1* deficiency activated ER stress and PCD at the cellular level.

MATERIALS AND METHODS

Virus-induced gene silencing

Virus-induced gene silencing of *NbBPS1* was performed as described previously (Kang et al., 2008b).

Realtime quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Realtime quantitative RT-PCR was performed with RNA isolated from the fourth leaf above the infiltrated leaf as described (Ahn et al., 2011). To detect *BLPs*, *HSP70*, and *Bax Inhibitor-1 (BI1)* transcripts, the following primers were designed, based on the published tobacco and *N. benthamiana* cDNA sequences: *BLP1* (X60060; 5'-aacaccgtcatccaacc-3' and 5'-tgg-tctgtctgtcttggtaa-3'), *BLP2* (X60059; 5'-atcctgatgagcagtgct-3' and 5'-cctctccgctaaggatacca-3'), *BLP3* (X60061; 5'-gagacag-cogtaaaggaagc-3' and 5'-ctcttctcagcgtctgtg-3'), *BLP4* (X60057; 5'-tctttccacaaatggagacac-3' and 5'-tccataatcctctgtgcaagtc-3'), *BLP5* (X60058; 5'-tgtgtatcagagatctggtggag-3' and 5'-tcattcc-tcgtggattcct-3'), *BI1* (AF390556; 5'-gaagaatgcattccgacaagg-3' and 5'-ccgcttatgcattgttctcc-3'), *HSP70* (GQ354819; 5'-ggat-tactgatactgagcgtct-3' and 5'-ttgatagggctcatagctactga-3'), and α -tubulin (AJ421411; 5'-atggcttctgctcattgtt-3' and 5'-cacag-cagcattgacatct-3').

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed as described (Ahn et al., 2011). Leaves from TRV control and TRV:BPS1(C) VIGS plants were fixed and treated with DNase-free RNase A (30 μ g/ml; Promega) and Proteinase K (20 μ g/ml) for 5 min before the TUNEL assay. The assay was carried out according to the manufacturer's manual (Promega) and counterstained with DAPI solution (0.1 μ g/ml DAPI, 50 mM NaCl, 5 mM EDTA, 10 mM mercaptoethylamine, and 10 mM Tris-HCl [pH 7.4]) for 5-10 min before examination with confocal microscopy (Zeiss LSM510). For a positive control, leaves from the TRV plants were treated with RQ1 RNase-Free DNase (Promega) for 15 min at 37°C before TUNEL assay according to the manual (Promega).

Measurement of *in vivo* H₂O₂ levels

H₂DCFDA staining and fluorescence measurement were carried out as described previously (Kim et al., 2006).

Agrobacterium-mediated transient expression of GFP:bZIP28

The GFP:bZIP28 fusion construct between *GFP* and *Arabidopsis bZIP28* (At3g10800) under the control of the CaMV35S promoter was generated as described (Liu et al., 2007). Agroinfiltration was carried out as described (Voinnet et al., 2003). Agrobacterial cultures (GV3101) containing the *GFP:bZIP28* construct fused to the CaMV35S promoter were adjusted to OD₆₀₀ = 0.6 in 2-(N-morpholino)ethanesulfonic acid (MES) buffer. The suspension was incubated with acetosyringone for 2-3 h at a final concentration of 150 μ M and infiltrated into leaves of TRV and TRV:BPS1(C) plants at 15 days after infiltration (DAI). Protoplasts were prepared from the infiltrated leaves collected from independent plants at 48 h post-infiltration and GFP:bZIP28 fluorescence localization was examined by fluorescence micro-

scopy and confocal laser scanning microscopy.

Lysotracker staining and GFP:ATG8e expression

Lysotracker Red (LTR) fluorescence indicative of autophagy activity was examined by using confocal microscopy (Zeiss LSM510) as described (Liu et al., 2005). Leaf protoplasts isolated from VIGS plants were stained with 1 μ M LTR (Molecular Probes) and kept for 1 min in darkness before visualization with confocal microscopy. Quantification of LTR-stained autophagosome-like structures was carried out using confocal microscopy.

The GFP:ATG8e fusion construct between *GFP* and *Arabidopsis ATG8e* (At2g45170) under the control of the CaMV35S promoter was generated as described (Ahn et al., 2011; Contento et al., 2005). Agrobacterial cultures (C58C1) containing the GFP:ATG8e construct were adjusted to OD₆₀₀=0.6 in MES buffer (10 mM MES [pH 7.5], 10 mM MgSO₄). The suspension was incubated with acetosyringone for 2-3 h at a final concentration of 150 μ M and infiltrated into leaves of VIGS plants. Protoplasts were prepared from the infiltrated leaves at 48 h post-infiltration and localization of the GFP fluorescence was examined by using confocal microscopy.

Transmission electron microscopy

Tissue sectioning and transmission electron microscopy were carried out using the fourth or fifth leaf above the infiltrated leaf of TRV, TRV:BPS1(C), and TRV:BPS1(U) lines as described by Ahn et al. (2004).

RESULTS AND DISCUSSION

Cell death phenotypes of TRV:NbBPS1 VIGS plants

Silencing of *NbBPS1* by VIGS with TRV:BPS1(U) and TRV:BPS1(C) constructs resulted in growth arrest, abnormal leaf development, and cell death (Figs. 1A-1C). Necrotic lesions developed in leaves at 15-16 days after infiltration (DAI), leading to premature plant death after 45 DAI (Fig. 1B). The intense blue pigment in Evans blue-stained leaves of TRV:BPS1(C) plants indicates localized cell death (Fig. 1D).

Analysis of programmed cell death phenotypes

We examined nuclear DNA fragmentation as a PCD marker in TRV:BPS1(C) leaf cells using the TUNEL assay. The TUNEL assay detects single- and double-strand DNA breaks by addition of fluorescent nucleotides to free 3' DNA termini (Gavrieli et al., 1992). TRV:BPS1(C) leaves exhibited strong fluorescent TUNEL staining in the epidermal nuclei based on confocal microscopy, suggesting single- or double-stranded DNA breaks in the nuclei (Fig. 2A). The staining was not detected in control TRV plants unless the plants had been treated with DNase (Fig. 2A). These results indicate that VIGS-mediated *NbBPS1* down-regulation causes PCD in *N. benthamiana*. During apoptosis in animal cells, activation of the cell death pathway is initiated by modification of the mitochondrial membrane potential. We have previously shown that the mitochondrial membrane potential was significantly reduced (about 4-fold) in the leaf protoplasts of the *NbBPS1* VIGS lines using TMRM (Tetramethylrhodamine methyl ester) fluorescent probes (Kang et al., 2008b).

To test whether reactive oxygen species (ROS) were produced in the TRV:BPS1(C) leaf cells undergoing PCD, we prepared leaf protoplasts from TRV or TRV:BPS1(C) plants and incubated the protoplasts with H₂DCFDA, a membrane-permeable indicator of ROS. Activation of the indicator depends on the presence of H₂O₂ and produces a green fluorescent signal

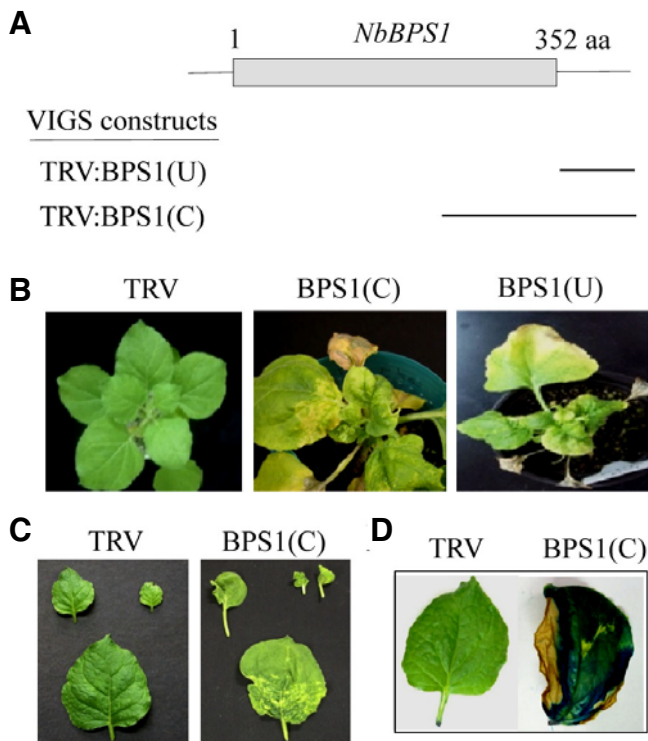


Fig. 1. VIGS constructs, phenotypes, and suppression of the *NbBPS1* transcripts. (A) Schematic drawing of *NbBPS1* structure and two VIGS constructs, TRV:BPS1(U) and TRV:BPS1(C), containing different *NbBPS1* cDNA fragments (indicated by bars). The gray box indicates the coding region of *NbBPS1*. (B) Cell death phenotypes of the *NbBPS1* VIGS plants. Photographs of the plants were taken at 25 days after infiltration (DAI). (C) Abnormal leaf development and lesion formation in TRV:BPS1(C) plants at 15 DAI. (D) Evans blue staining visualizes leaf tissues undergoing cell death in TRV:BPS1(C) lines.

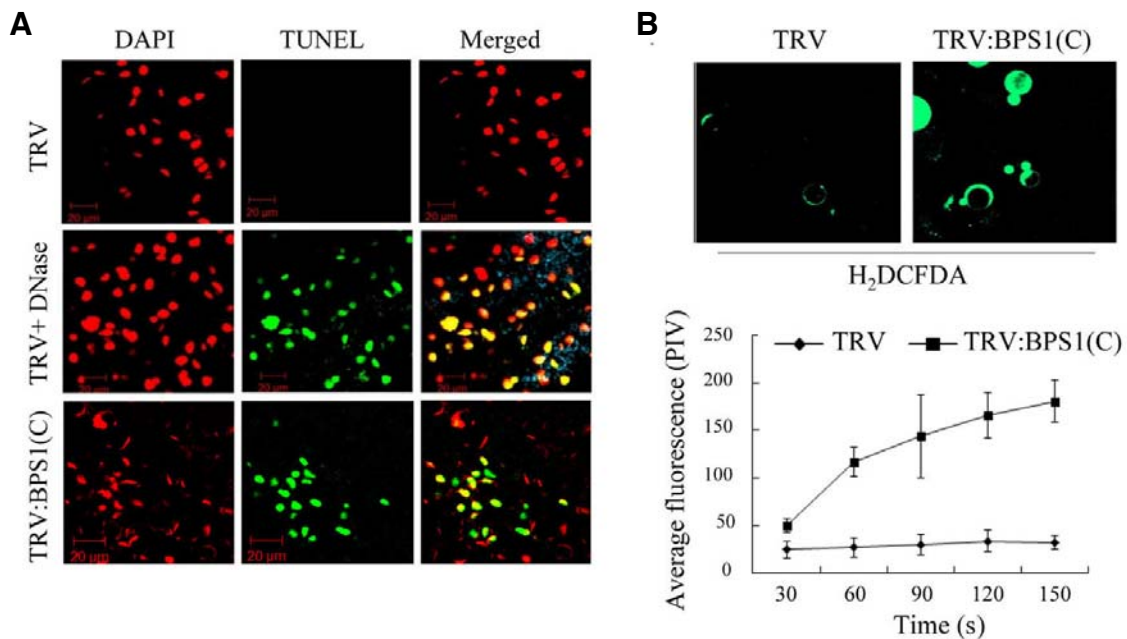


Fig. 2. Phenotypes of programmed cell death. (A) Confocal images of the TUNEL assay in leaves of TRV control and TRV:BPS1(C) plants at 15 DAI. Leaves were counterstained with DAPI. As a positive control, fixed leaf material of TRV samples was subjected to DNase treatment. Scale bars = 20 μm . (B) ROS production. Leaf protoplasts from TRV control and TRV:BPS1(C) plants were incubated with the ROS indicator H_2DCFDA (2 μM) (top). H_2DCFDA is a ROS indicator that becomes fluorescent when oxidized by ROS within the cell. Fluorescence of protoplasts from the VIGS lines was quantified by pixel intensity (bottom). Data points represent means \pm SD of 30 individual protoplasts. PIV, pixel intensity values.

(Fig. 2B). The rate of accumulation of fluorescent H_2DCFDA in TRV:BPS1 protoplasts was approximately six times greater

than that of the TRV control protoplasts, suggesting involvement of ROS in the cell death program (Fig. 2B).

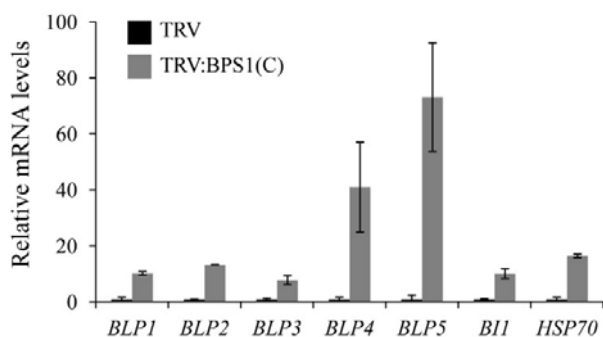


Fig. 3. Induction of ER stress-related genes in TRV:BPS1 plants. Real-time quantitative RT-PCR analyses were performed to determine transcript levels of ER stress-related genes in TRV and TRV:BPS1(C) leaves at 15 DAI. Each value represents the mean \pm SD of three replicates per experiment. The α -tubulin mRNA level was used as a control.

Expression of ER stress-related genes

NbBPS1 is localized in the ER in *N. benthamiana* (Kang et al., 2008b). The cell death phenotype of the *NbBPS1* VIGS plants observed may indicate that *NbBPS1*-deficient cells were subjected to ER stress. Previously, transcriptome analyses revealed that transcription of *BiPs*, *BI1* (*Bax-Inhibitor-1*), and *HSP70* was up-regulated in response to tunicamycin-induced ER stress in *Arabidopsis* (Urade, 2007; 2009; Watanabe and Lam, 2008). Functions of these genes during ER stress have been reported: overexpression of BLPs alleviated ER stress in tobacco (Leborgne-Castel et al., 1999) and BI-1 modulated ER

stress-mediated PCD in *Arabidopsis* (Watanabe and Lam, 2008). Thus, we examined transcript levels of those ER stress-related genes in TRV and TRV:BPS1(C) lines (Fig. 3). Realtime quantitative RT-PCR revealed that the transcript levels of *N. benthamiana* *BLP1* (*BiP-like protein 1*), *BLP2*, *BLP3*, *BLP4*, *BLP5*, *BI1*, and *Hsp70* genes were all induced to a much higher level in TRV:BPS1(C) leaves at 15 DAI than in TRV control leaves (Fig. 3). Since *BLPs* encoding ER-localized chaperones and *BI1* have been used as marker genes for ER stress and UPR activation in plants (Martinez and Chrispeels, 2003; Watanabe and Lam, 2008; Zuppini et al., 2004), our results suggest that *NbBPS1* deficiency induces ER stress in *N. benthamiana*.

Processing of bZIP28 transcription factor indicating ER stress

In *Arabidopsis*, tunicamycin-induced ER stress causes proteolytic processing of bZIP28 and bZIP60, ER membrane-associated bZIP transcription factors, and translocation of their released N-terminal domains to the nucleus (Iwata and Koizumi, 2005; Liu and Howell, 2010; Liu et al., 2007a). In the nucleus, the N-terminal domains of the bZIP factors control transcriptional induction of ER stress response genes (Liu and Howell, 2010). A similar processing and nuclear translocation of *Arabidopsis* bZIP17 occurs in response to salt stress (Liu et al., 2007b). To confirm that *NbBPS1* deficiency causes ER stress, we examined localization of bZIP28 in control TRV and TRV:BPS1(C) lines (Fig. 4). A GFP fusion protein of bZIP28, GFP:bZIP28, was transiently expressed in TRV and TRV:BPS1(C) leaves at 15 DAI by agro-infiltration. Protoplasts (mostly from mesophyll cells) were isolated from the infiltrated leaves and examined by confocal laser scanning microscopy (Fig. 4A). In

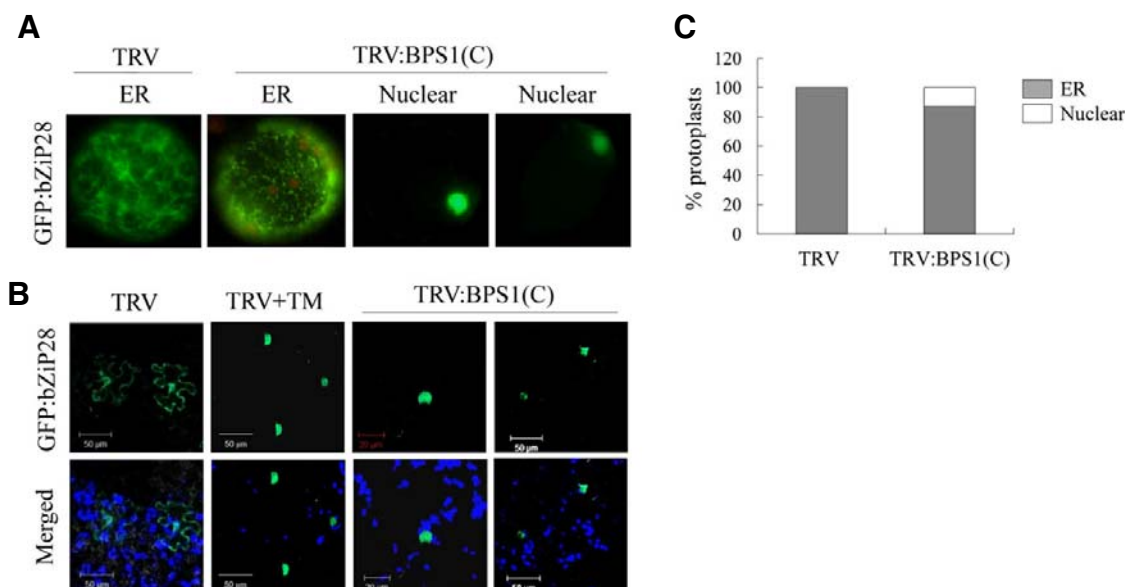


Fig. 4. Subcellular localization of GFP:bZIP28. (A) Protoplasts were isolated from TRV and TRV:BPS1(C) plants (15 DAI) infiltrated with *Agrobacterium* containing *GFP:bZIP28*, and localization of fluorescent signals was examined by fluorescence microscopy. Representative images of ER and nuclear localization of GFP:bZIP28 in the protoplasts are shown. (B) TRV control, tunicamycin (TM)-treated TRV, and TRV:BPS1(C) plants (15 DAI) were infiltrated with *Agrobacterium* containing *GFP:bZIP28* construct. After 24 h, localization of GFP fluorescent signals in leaf epidermal cells was observed by confocal microscopy (*top*). Merged images of GFP:bZIP28 fluorescence and chlorophyll autofluorescence (pseudo-colored blue; mostly from underlying mesophyll cells) are also shown (*bottom*). Representative images are shown. (C) The frequency of ER and nuclear patterns of GFP:bZIP28 localization in the protoplasts ($n = 200$) from TRV and TRV:BPS1(C) lines shown in (A).

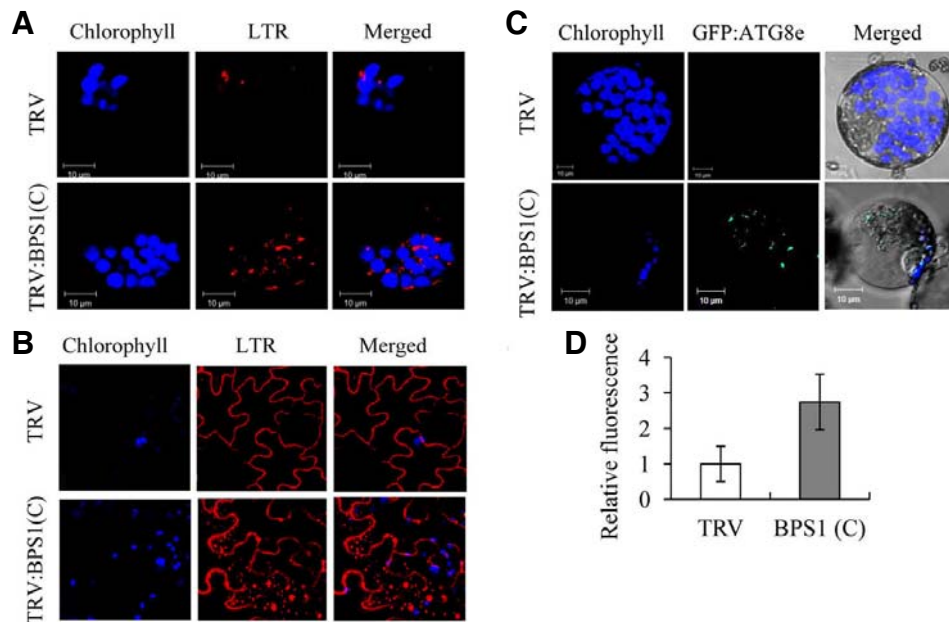


Fig. 5. Activation of autophagy. (A) LysoTracker Red (LTR) staining of leaf protoplasts isolated from TRV and TRV:BPS1(C) lines at 15 DAI. LTR-stained punctuate autophagosome-like structures, chloro-phyll autofluorescence (pseudo-colored blue), and merged images were observed by confocal laser scanning microscopy. (B) LTR staining of leaf epidermal cells of TRV and TRV:BPS1(C) lines at 15 DAI. Note that LTR also stains the epidermal cell wall. (C) Confocal microscopy of transiently expressed GFP:ATG8e in leaf protoplasts isolated from TRV and TRV:BPS1(C) lines at 15 DAI. (D) LTR-derived red fluorescence of the protoplasts shown in (A) was quantified by confocal microscopy. Data points represent means \pm SD of 30 individual protoplasts.

the TRV protoplasts, GFP:bZIP28 displayed a network pattern of green fluorescence throughout the cytoplasm, indicating localization of bZIP28 to the ER. This pattern of bZIP28 localization is consistent with the results by Liu et al. (2007a). Most of the protoplasts from TRV:BPS1(C) leaves also exhibited the network-like pattern, but 9% of the protoplasts ($n = 200$) showed strong GFP fluorescence only in the nucleus, suggesting relocation of the bZIP28 factor to the nucleus (Figs. 4A and 4C). Confocal microscopy of the leaf epidermal cells revealed green fluorescent signals of GFP:bZIP28 in the vicinity of the plasma membrane and the nucleus in both TRV and TRV:BPS1(C) leaves (Fig. 4B). However, some epidermal cells in TRV:BPS1(C) lines clearly exhibited strong fluorescent signal only in the nucleus, similar to the pattern in tunicamycin-treated TRV leaf cells (Fig. 4B). Since nuclear accumulation of GFP:bZIP28 was not observed in TRV cells, these results further support that *NbBPS1* silencing induces the ER stress response.

Induction of autophagy

Autophagy is a conserved catabolic pathway in eukaryotic cells that controls recycling of bulk cytoplasmic materials from proteins to whole organelles (Bassham, 2007; Lum et al., 2005). During autophagy, cytoplasm is enclosed in small vesicles (autophagosomes) and delivered to the lysosome or lytic vacuole for degradation. Because ER stress can activate autophagy (Ding and Yin, 2008; Høyer-Hansen and Jäättelä, 2007; Sakaki and Kaufman, 2008), we examined the autophagic activity of TRV:BPS1(C) and TRV control plants at 15 DAI (Fig. 5). Epidermal cells and leaf protoplasts from the VIGS plants were stained with LysoTracker Red (LTR) dye as a probe to detect autolysosome structures (Figs. 5A, 5B, and 5D). The LTR dye labels acidic organelles such as autolysosomes, but it also

stains the cell wall in epidermal cells (Hofius et al., 2009; Liu et al., 2005). Many LTR-stained particles were observed in the cytosol of the epidermal cells and the protoplasts from *NbBPS1*-silenced plants, but were rarely observed in the cytoplasm of TRV control (Figs. 5A and 5B). The LTR fluorescence in the protoplasts was quantified: TRV:BPS1(C) protoplasts exhibited almost three times the level of fluorescence of TRV (Fig. 5D).

Autophagy activity was further monitored by transient expression of a GFP:ATG8e fusion protein, which has been used to visualize autophagy in plant cells (Contento et al., 2005). GFP:ATG8e was transiently expressed in the VIGS plants (15 DAI) by agroinfiltration. After 2 days, protoplasts were generated from the leaves and examined by confocal microscopy (Fig. 5C). GFP fluorescence was evident as multiple punctuate structures in TRV:BPS1(C) protoplasts, but was not detectable in TRV protoplasts (Fig. 5C; Supplementary Fig. 1A). Transient expression of the *GFP:ATG8e* transcript in the infiltrated leaves of TRV control and TRV:BPS1(C) lines was determined by using RT-PCR (Supplementary Fig. 1B). These confocal microscopy observations indicate that *NbBPS1* silencing results in activation of autophagy.

Ultrastructural analyses using transmission electron microscopy

Using transmission electron microscopy, cell ultrastructure of TRV:BPS1 lines was examined compared with TRV control plants at 15 DAI. Leaf cells of TRV:BPS1(C) and TRV:BPS1(U) lines accumulated a large number of small vesicles or vacuoles near the plasma membrane (Figs. 6C-6H) compared to TRV control plants (Figs. 6A and 6B). Dramatic degeneration of cellular ultrastructure was evident in TRV:BPS1 lines. High magnification showed that most membrane vesicles had double or

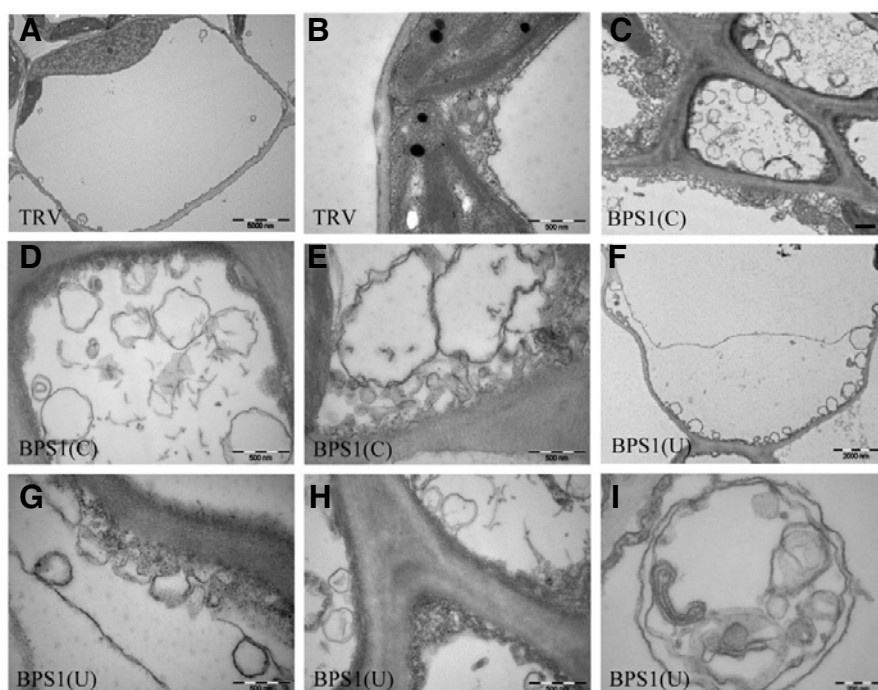


Fig. 6. Accumulation of vesicles in TRV:BPS1 leaf cells. Transmission electron micrographs of leaf transverse sections from TRV (A, B), TRV:BPS1(C) (C-E), and TRV: BPS1(U) (F-I) lines at 15 DAI. Autophagosome-like structures were observed near plasma membranes in TRV:BPS1 lines (C-I). Scale bars = 5 μm (A); 0.5 μm (B-H); 0.2 μm (I).

multiple membrane boundaries, a morphological feature of autophagosomes (Fig. 6I).

Previously, *NbBPS1* VIGS led to severe developmental abnormalities and cell death in *N. benthamiana* (Kang et al., 2008b). *NbBPS1* shows no homology to any known proteins. *NbBPS1* localization in the ER has been shown (Kang et al., 2008b), but its biochemical function in the ER remains unclear. Our results in this study suggest that severe deficiency of *NbBPS1* in *N. benthamiana* induces ER stress, autophagy, and PCD, leading to premature death of the plants. *NbBPS1* dysfunction may result in accumulation of misfolded proteins in the ER, either directly or indirectly, leading to the ER stress. Induction of autophagy in the *NbBPS1* VIGS plants can be explained by the findings that ER stress is a potent trigger of autophagy, and that the phenomenon is conserved from yeast to mammals (Ding and Yin, 2008; Høyer-Hansen and Jäättelä, 2007; Sakaki and Kaufman, 2008). Further study should illustrate the molecular function of *NbBPS1* and its connection to the regulatory mechanism of plant growth and development.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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