Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(9), 1382–1397 DOI: 10.1111/mpp.12369

Endoplasmic reticulum stress responses function in the HRT-mediated hypersensitive response in *Nicotiana benthamiana*

JU YEON MOON[1,2], JEONG HEE LEE[1], CHANG-SIK OH[3], HONG-GU KANG[4] AND JEONG MEE PARK[1,2,*]

[1]*Molecular Biofarming Research Center, KRIBB, Daejeon 305-600, South Korea*
[2]*Department of Biosystems and Bioengineering, UST, Daejeon 305-350, South Korea*
[3]*Department of Horticulture, Kyung Hee University, Yongin 446-701, South Korea*
[4]*Department of Biology, Texas State University, San Marcos, TX 78666, USA*

SUMMARY

HRT is a plant coiled-coil, nucleotide-binding and leucine-rich repeat (CC-NB-LRR) disease resistance protein that triggers the hypersensitive response (HR) on recognition of *Turnip crinkle virus* (TCV) coat protein (CP). The molecular mechanism and significance of HR-mediated cell death for TCV resistance have not been fully elucidated. To identify the genes involved in HRT/TCV CP-mediated HR in *Nicotiana benthamiana*, we performed virus-induced gene silencing (VIGS) of 459 expressed sequence tags (ESTs) of pathogen-responsive *Capsicum annuum* genes. VIGS of *CaBLP5*, which encodes an endoplasmic reticulum (ER)-associated immunoglobulin-binding protein (BiP), silenced *NbBiP4* and *NbBiP5* and significantly reduced HRT-mediated HR. The induction of ER stress-responsive genes and the accumulation of ER-targeted BiPs in response to HRT-mediated HR suggest that ER is involved in HR in *N. benthamiana*. *BiP4/5* silencing significantly down-regulated *HRT* at the mRNA and protein levels, and affected *SGT1* and *HSP90* expression. Co-expression of *TCV CP* in *BiP4/5*-silenced plants completely abolished *HRT* induction. Transient expression of *TCV CP* alone induced selected ER stress-responsive gene transcripts only in *Tobacco rattle virus* (TRV)-infected plants, and most of these genes were induced by HRT/TCV CP, except for *bZIP60*, which was induced specifically in response to HRT/TCV CP. TCV CP-mediated induction of ER stress-responsive genes still occurred in *BiP4/5*-silenced plants, but HRT/TCV CP-mediated induction of these genes was defective. Tunicamycin, a chemical that inhibits protein *N*-glycosylation, inhibited HRT-mediated HR, suggesting that ER has a role in HR regulation. These results indicate that BiP and ER, which modulate pattern recognition receptors in innate immunity, also regulate R protein-mediated resistance.

Keywords: BiP, ER stress, HR cell death, HRT, R protein, TCV CP.

*Correspondence: Email: jmpark@kribb.re.kr

INTRODUCTION

Effector-triggered immunity (ETI) is one of the sophisticated disease resistance mechanisms that plants have evolved to protect themselves against a wide range of pathogens. ETI is generally triggered when a plant resistance (R) protein directly or indirectly recognizes a corresponding pathogen effector protein (Gassmann and Bhattacharjee, 2012). ETI triggers diverse cellular responses, including an oxidative burst, the expression of defence-related genes and the hypersensitive response (HR) (Hammond-Kosack and Parker, 2003). HR triggers rapid and localized cell death at infection sites, and is a potent resistance response that limits pathogen growth in plants.

Many R proteins encoded by plant genomes belong to the nucleotide-binding and leucine-rich repeat (NB-LRR) protein family. The NB-LRR class of R proteins can be further categorized into two groups depending on whether they have a Toll/interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain at the N-terminus (Collier and Moffett, 2009). Several important signalling components involved in R protein function have been identified and extensively characterized. R proteins with TIR or CC domains require EDS1 (enhanced disease susceptibility 1) or NDR1 (non-race-specific disease resistance 1), respectively, for downstream signal transduction (Aarts *et al.*, 1998). Several co-chaperone proteins, such as heat shock protein 90 (HSP90), SGT1 (suppressor of G2 allele of Skp1) and RAR1 (required for Mla12 resistance), are critical for the stabilization of R proteins in plant cells (Hubert *et al.*, 2009; Shirasu, 2009). CRT/MORC1 (compromised recognition of TCV/Microchidia 1) was recently identified as a molecular modulator that regulates diverse R protein functions and mostly localizes to endomembrane systems in plants (Kang *et al.*, 2010).

The HSP70 protein family contains a large group of well-characterized chaperones. In *Arabidopsis*, cytosolic and nuclear heat shock cognate 70 (HSC70) proteins interact with SGT1 and regulate immune responses (Noel *et al.*, 2007). Another subclass of HSP70s, the luminal binding proteins (BiPs; also known as Glucose Regulated Protein 78, GRP78), is targeted to the endoplasmic reticulum (ER) in yeast and mammals (Kohno *et al.*, 1993; Lee, 1992). In yeast, BiP associates with nascent polypeptides and

functions in the translocation and folding of newly synthesized proteins (Zimmermann *et al.*, 2011). Plant BiPs function similarly to other eukaryotic BiPs; a tobacco BiP can complement a yeast temperature-sensitive *kar2* mutant (Denecke *et al.*, 1991), and *BiP* overexpression in tobacco alleviates ER stress resulting from tunicamycin (Tm) treatment, an inhibitor of *N*-linked protein glycosylation (Leborgne-Castel *et al.*, 1999). However, plants have multiple *BiP* genes, unlike other eukaryotes (Denecke *et al.*, 1991; Kalinski *et al.*, 1995; Wrobel *et al.*, 1997). Although some studies have reported that plant BiPs are involved in environmental stress responses, the functional specificity and redundancy among plant BiP family members remain to be elucidated.

Plant BiPs are involved in a wide range of functions, including immune responses. *AtBiP2* silencing in *Arabidopsis* attenuates NPR1 (nonexpressor of pathogenesis-related genes 1)-dependent secretion of PR1 (pathogenesis-related 1) proteins, indicating that AtBiP2 is involved in the efficient induction of systemic acquired resistance (SAR) (Wang *et al.*, 2005). *BiP3* overexpression in rice reduces innate immunity mediated by receptor-like kinase Xa21 (Park *et al.*, 2010, 2014a), suggesting that this protein negatively regulates innate immunity. Yeast ERD2 (endoplasmic reticulum retention defective 2) homologues, which are ER luminal protein receptors, have a critical role in the regulation of HR induction in *N. benthamiana*; silencing of the tobacco homologues *NtERD2a* and *NtERD2b* in *N. benthamiana* increases HR induced by R proteins, such as tobacco N and tomato Cf9 and Pto (Xu *et al.*, 2012). Recent work has shown that overexpression of tobacco *BiP4* or *BiP2* in soybean enhances non-host HR triggered by *Pseudomonas syringae* pv. *tomato*, further confirming that plant BiP proteins have important roles in plant immunity (Carvalho *et al.*, 2014).

The *Arabidopsis* R protein HRT, which confers disease resistance to *Turnip crinkle virus* (TCV), belongs to the HRT/RPP8 (hypersensitive response of TCV/recognition of Peronospora parasitica 8) protein family, whose members contain CC-NB-LRR domains (Cooley *et al.*, 2000). On recognition of the TCV coat protein (CP), HRT triggers HR in inoculated leaves and local and systemic defence responses. A recessive allele, designated *rrt*, is required for HRT-mediated HR and complete resistance to TCV infection (Cooley *et al.*, 2000). The requirement for *rrt* in resistance can be bypassed by increasing the *HRT* levels via the exogenous application of salicylic acid (SA) or by transgenic *HRT* overexpression. A limited number of host proteins required for *HRT*/*rrt*-mediated resistance, including EDS1, SID2 (salicylic acid induction-deficient 2), PAD4 (protein arginine deiminase 4), EDS5 and CRT1/MORC1, have been identified in *N. benthamiana* transient expression studies and *Arabidopsis* mutant analyses (Chandra-Shekara *et al.*, 2004; Kang *et al.*, 2008).

In this study, we used virus-induced gene silencing (VIGS) to identify new signalling components associated with HRT/TCV CP-mediated HR in *N. benthamiana*. We found that transient co-expression of *HRT* and its elicitor *TCV CP* induced cell death in *N. benthamiana*. We used this system in conjunction with VIGS, and showed that the silencing of *BiP4* and *BiP5* genes, which encode ER-resident HSP70 proteins of *N. benthamiana*, attenuates HRT-mediated HR by down-regulating *HRT* mRNA levels and protein expression. This work shows that ER stress responses play a role in HRT-mediated HR induction.

RESULTS

Agrobacterium-mediated TCV CP and HRT co-expression induces HR-like cell death in *N. benthamiana*

On recognition of TCV CP, transgenic *Arabidopsis* overexpressing *HRT* undergoes HR-like cell death (Cooley *et al.*, 2000). To investigate whether the ectopic expression of HRT protein in conjunction with TCV CP can specifically induce HR in other plant species, we transiently expressed both proteins in *N. benthamiana*. When either HRT or TCV CP alone was constitutively expressed, no cell death was detected; however, when HRT or haemagglutinin (HA)-tagged HRT was co-expressed with TCV CP, HR-like cell death was induced within 48 h after agroinfiltration (Figs 1A and S1, see Supporting Information). The extent of cell death was comparable with that in plants co-expressing Pto and AvrPto, which are known to trigger cell death in *N. benthamiana* and were used as a positive control (Oh *et al.*, 2010). The visual cell death phenotype was verified biochemically by quantitative measurements of ion leakage from infiltrated leaf discs, which occurs during plant cell death (Fig. 1B) (Tamagnone *et al.*, 1998). To determine whether this specific recognition is conserved in *N. benthamiana*, we co-expressed HRT and a mutant TCV CP (D4N), which is known to escape HRT recognition (Zhao *et al.*, 2000). Unlike the effects of HRT/TCV CP, co-expression of HRT/TCV CP (D4N) did not induce HR-like cell death symptoms (Fig. 1A). Consistently, the ion leakage measurements indicated that there was no cell death response in *N. benthamiana* leaves expressing HRT alone or HRT with TCV CP (D4N) (Fig. 1B). These combined results indicate that HRT does not have autoactivation activity, but causes HR-like cell death by specific recognition of TCV CP in *N. benthamiana*.

BiP4/5 silencing in *N. benthamiana* disrupts cell death triggered by the interaction between HRT and TCV CP

To identify signalling components that modulate HRT/TCV CP-mediated HR in *N. benthamiana*, we performed *Tobacco rattle virus* (TRV)-mediated VIGS of 459 expressed sequence tags (ESTs) from *Capsicum annuum*, which were induced in response to pathogen challenge (Lee *et al.*, 2013). Silencing of one of these ESTs consistently reduced HR induction caused by HRT/TCV CP in *N. benthamiana* (Fig. 2A). This EST, which appeared to lack the native N-terminal region, corresponds to a UniGene (*cacn15824*)

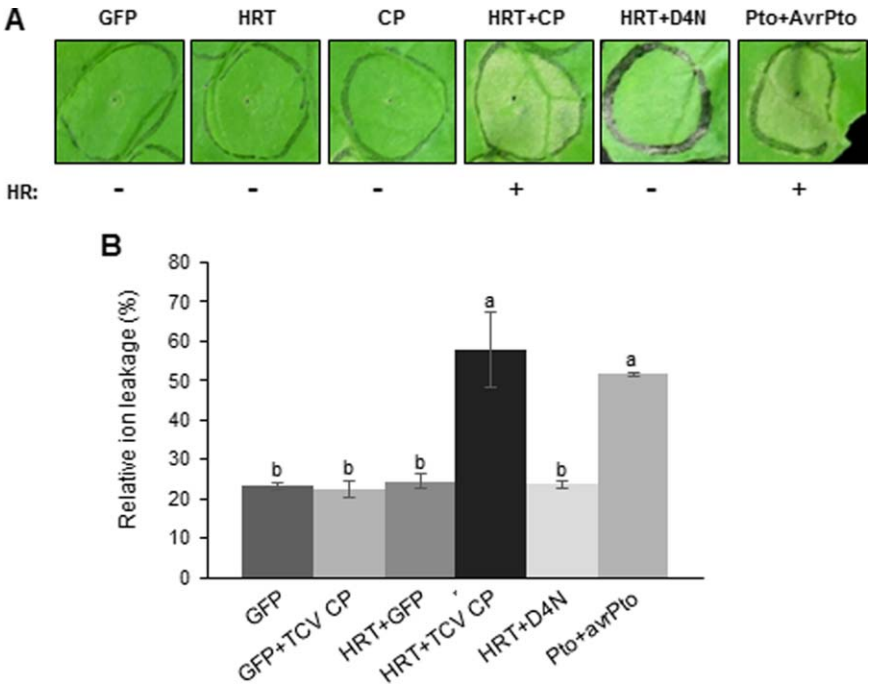

Fig. 1 Co-expression of *HRT* and *Turnip crinkle virus* coat protein (TCV CP) induced the hypersensitive response (HR) in *Nicotiana benthamiana*. (A) HR induction on leaves of *N. benthamiana* after transient expression of the indicated genes. Photographs were taken at 3 days post-infiltration (dpi). GFP, green fluorescent protein; +, HR; −, no HR. (B) Electrolyte leakage assay. Measurements of ion conductivity were performed at 3 dpi as in (A). Error bars represent the standard deviation of duplicate measurements calculated from 40 leaf discs (diameter, 1 cm) per construct. Means with the same letter are not significantly different (*t*-test, $P < 0.05$).

with 34 ESTs. This gene was named *CaBLP5* (*Capsicum annum* BiP5-like protein; GenBank accession no. AGS42239) because it had 97% amino acid identity with tomato SlBiP5 protein, which is a luminal binding protein belonging to the HSP70 family of ER-targeted proteins (Denecke *et al.*, 1991). A domain search using InterProScan revealed that, like SlBiP5, the CaBLP5 protein contains an N-terminal signal peptide (SP), an ATPase domain, a C-terminal substrate binding domain and an ER retention motif (Denecke *et al.*, 1991).

The *HSP70* family contains many genes, including *BiP*. Although HSP70 proteins are highly similar, their variable N- and C-termini are responsible for their subcellular localization and intra- or intermolecular interactions (Lin *et al.*, 2001). The GenBank database identified 26 proteins closely related to CaBLP5 from diverse plant species. Based on the phylogenetic analysis of these homologues, CaBLP5 was grouped together with tomato SlBiP5 and potato StBiP5-L (Fig. S2, see Supporting Information).

To analyse the specificity of TRV2-*CaBLP5*-mediated gene silencing, we searched for *CaBLP5* homologues by performing a BLAST search in the *N. benthamiana*_ transcriptome_v3_ unigenes 95 database (http://benth-web-pro-1.ucc.usyd.edu.au/blast/search.php). This analysis retrieved six *NbBiP* homologues, and their nucleotide sequences aligned with *CaBLP5* (Table S1 and Fig. S3, see Supporting Information). A sequence of more than 21 nucleotides with 100% identity between the trigger and the target sequence is required to cause gene silencing during VIGS (Liu *et al.*, 2002a). Therefore, we examined whether the six *NbBiP* sequences have a 21-nucleotide sequence with 100% identity with TRV2-*CaBLP5* (Fig. S3). Consequently, we found that TRV2-*CaBLP5* contains a stretch of 21 nucleotides with 100% identity with *Nbv3K645786225* and *Nbv3K645789686*, and multiple sites with more than 21-nucleotide stretches that are identical with *Nbv3K685813373*. By contrast, the other three *BiP* homologues, *Nbv3K585690033*, *Nbv3K585703505* and *Nbv3K765636570*, did not have a 21-nucleotide stretch with 100% identity to TRV2-*CaBLP5* (Fig. S3). A phylogenetic analysis based on the amino acid sequence alignment of NbBiPs with CaBLP5 and the tobacco and *Arabidopsis* BiPs places Nbv3K685813373 and Nbv3K645786225 (Nbv3K645789686 amino acid sequence is identical to that of Nbv3K645786225) in the same clade with CaBLP5 (Fig. S4 and S5, see Supporting Information). Nbv3K685813373 grouped with NtBiP5 is named NbBiP5. This clone shares 93% identity with *CaBLP5* at the nucleotide level (Table S1). The clone Nbv3K645786225 (identical to Nbv3K645789686), which shares 82% identity with *CaBLP5* at the nucleotide level, was named NbBiP4 based on homology with other BiP4s (Figs S3 and S4, Table S1).

To confirm the specificity of VIGS in *N. benthamiana*, we assessed the transcript levels of three *CaBLP5* homologues by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using specific primers for each *N. benthamiana BiP* gene in *GFP* (*green fluorescent protein*, negative control for VIGS) or *CaBLP5*-silenced plants after 3 weeks of TRV infection (Figs 2B, S3 and S4). The qRT-PCR results showed that the *CaBLP5* fragment targeted the *NbBiP5* and *NbBiP4* homologues (*Nbv3K685813373* and *Nbv3K645786225*), whereas *Nbv3K585690033* (encoding the

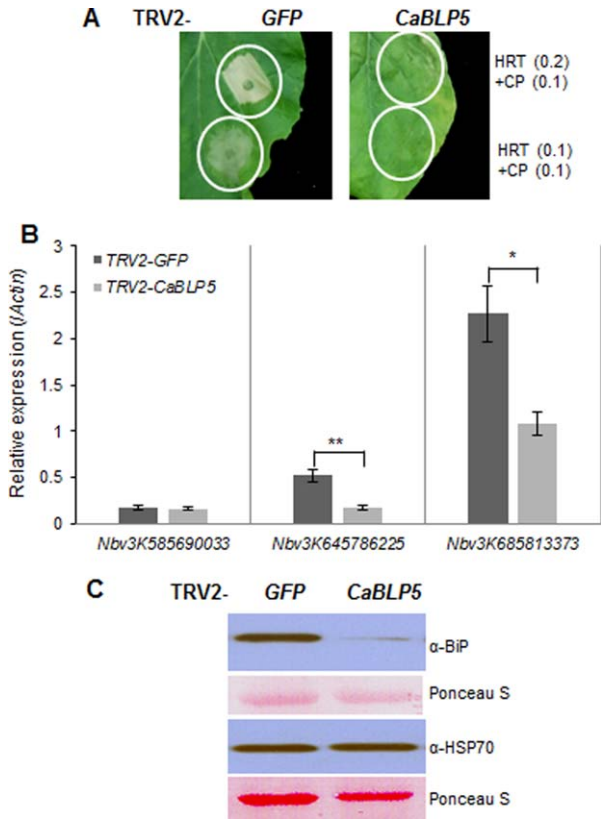

Fig. 2 Silencing of the *CaBLP5* homologue in *Nicotiana benthamiana* disrupts the HRT/*Turnip crinkle virus* coat protein (TCV CP)-mediated hypersensitive response (HR). (A) HR phenotypes induced by co-expression of *HRT* and *TCV CP* in *TRV2-GFP* or TRV2-*CaBLP5*-infected plants (GFP, green fluorescent protein; TRV, *Tobacco rattle virus*). Samples were photographed at 3 days post-infiltration (dpi). The numbers indicate *Agrobacterium* cell concentrations determined from the optical density at 600 nm (OD_{600}). (B) Suppression of *CaBLP5* homologous gene expression in *GFP*- or *CaBLP5*-silenced *N. benthamiana* plants. Error bars indicate the standard error of three replicates, and asterisks indicate a significant difference from the corresponding control (Student's *t*-test, $*P < 0.05$; $**P < 0.005$). (C) BiP protein levels in *GFP*- and *CaBLP5*-silenced *N. benthamiana* leaves. Total protein samples were extracted after 21 days of infection. Samples were probed with the indicated antibodies for each protein.

Arabidopsis BiP2 homologue) expression was not reduced in TRV2-*CaBLP5*-silenced leaves relative to the TRV2-*GFP* control (Figs 2B, S3 and S4). These results indicate that *NbBiP4* and *NbBiP5* are the targets of TRV2-*CaBLP5* gene silencing in *N. benthamiana*. To determine whether specific down-regulation of *BiP4/5* in silenced plants was reflected in encoded protein levels, we assessed ER-resident BiP and cytosol-resident HSP70 using two anti-HSP70 antibodies that differentially detect these homologous proteins (Ko *et al.*, 2007; Noel *et al.*, 2007). As anticipated, the levels of ER-resident BiP were greatly reduced, whereas cytosolic HSP70 levels were unchanged (Fig. 2C). These results further confirmed the specific down-regulation of ER-resident BiP proteins, but not cytosolic HSP70 proteins, by VIGS targeting of *CaBLP5*.

Co-expression of HRT and TCV CP induced a strong ER stress response in *N. benthamiana*

There are no reports that HRT-mediated HR is associated with ER stress responses, and so we assessed the possibility that HR suppression in *BiP4/5*-silenced plants was caused by the pleiotropic effects of silencing. To this end, we monitored the expression of a set of ER stress-responsive genes, including *BiP4/5*, in response to the expression of *HRT* and/or *TCV CP* in *N. benthamiana* leaves. We observed that transient *HRT* expression induced weak expression of ER stress-responsive genes compared with the expression of the *YFP* (*yellow fluorescent protein*) control, whereas TCV CP expression did not increase the expression of ER stress-responsive genes above that induced by the *YFP* control (Fig. 3A). However, we observed strong induction of ER stress-responsive genes by co-expression of *HRT* and *TCV CP*, indicating that the induction of ER stress-responsive genes specifically responds to the interaction between HRT and TCV CP. We also observed that, although *HRT* and *TCV CP* expression was driven by the 35S cauliflower mosaic virus (CaMV) promoter, *HRT* transcripts were strongly induced by the co-expression of *TCV CP*, whereas *TCV CP* transcripts were significantly reduced by the co-expression of *HRT* (Fig. 3A). These results suggest that the interaction between HRT and TCV CP affects the post-transcriptional regulation of their corresponding genes.

The induction of BiP protein expression was detected during HR progression (Fig. 3B). The expression of HRT/GFP and β-glucuronidase (GUS)/GFP was used as a negative control for HR. Following agroinfiltration to express HRT/GFP or GUS/GFP, BiP levels gradually increased over the next 24 h (Fig. 3B). Transient co-expression of HRT and TCV CP also increased BiP accumulation, but the induction occurred much earlier than that in the controls (Fig. 3B). These combined results suggest that ER stress responses are associated with the interaction of HRT and TCV CP, and that BiP is induced in response to HRT-mediated cell death.

Effect of SGT1 and HSP90 in conjunction with BiP4/5 in HRT-mediated HR

To study the interaction of BiP4/5 with HSP90 and SGT1, which are involved in R protein regulation as chaperone or co-chaperone (Bhattarai *et al.*, 2007; Hubert *et al.*, 2003; Lu *et al.*, 2003), we assessed HRT-mediated HR in *BiP4/5*-, *SGT1*- and *HSP90*-silenced *N. benthamiana* plants (Figs 4 and S7, see Supporting Information). Rx-mediated HR, which confers resistance to *Potato virus X* (PVX) via recognition of its CP (Bendahmane *et al.*, 2002; Cooley *et al.*, 2000), was used as an experimental control for SGT1 and HSP90 involvement in Rx/PVX-CP-mediated HR. HRT- and Rx-mediated HR was visibly disrupted in *CaBLP5*-, *HSP90*- and *SGT1*-silenced leaves, whereas normal HR was induced in TRV2-*GFP*

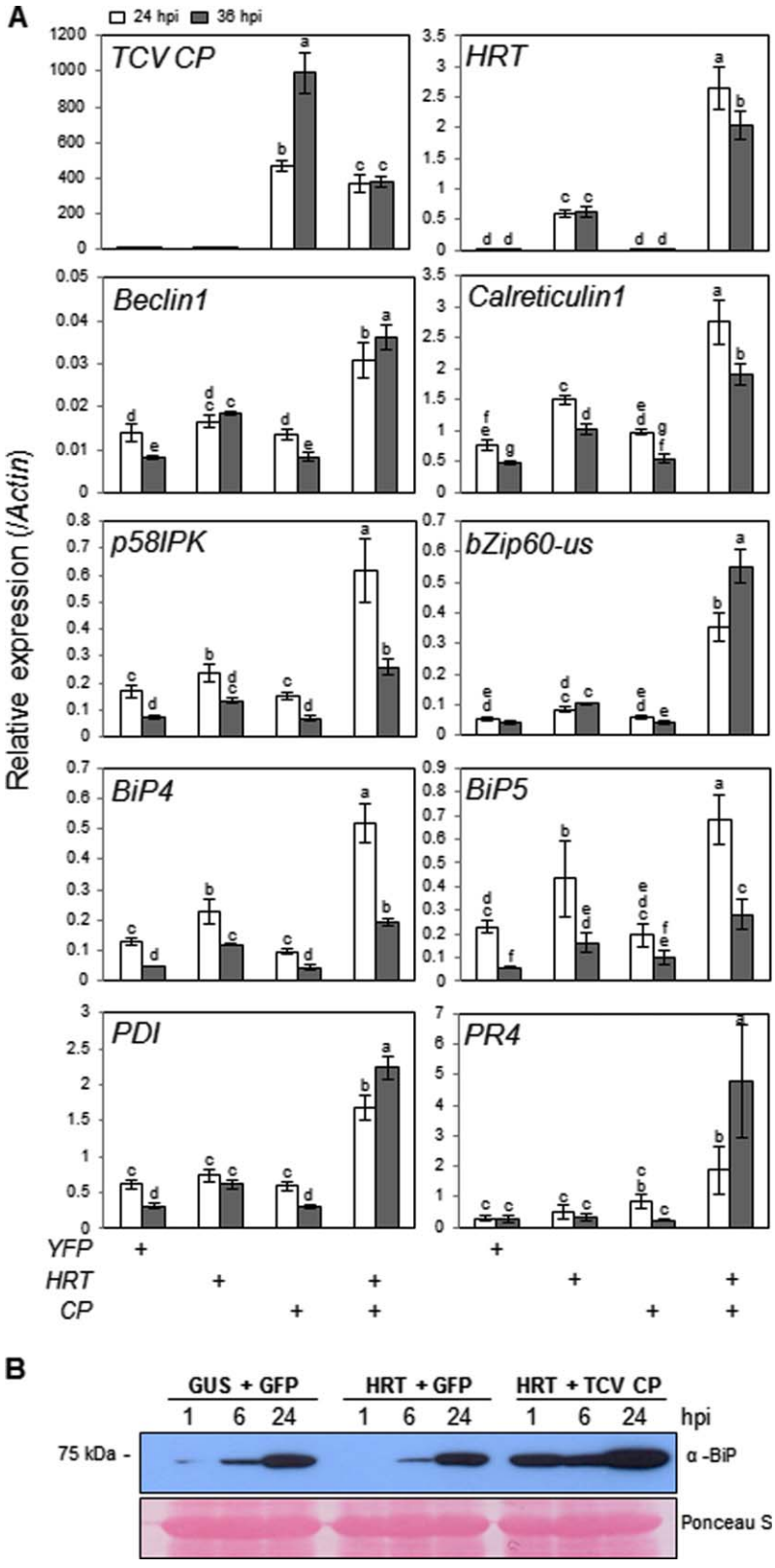

Fig. 3 Interaction between HRT and *Turnip crinkle virus* coat protein (TCV CP) induced the endoplasmic reticulum (ER) stress response. (A) The transcriptional levels of ER stress-responsive genes following the transient expression of TCV CP or HRT were assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The expression of *Calreticulin1*, *Beclin1*, *BiP4/5* and *PDI*, as markers for ER quality control (QC), and *bZIP60*, *p58*^{IPK} and *PR4*, as markers for ER stress, was analysed at 24 and 36 h post-infiltration (hpi). Error bars indicate standard deviation ($n = 3$). Bars with the same letter are not significantly different (*t*-test, $P < 0.05$). (B) BiP protein levels under the HRT-mediated hypersensitive response (HR) in *Nicotiana benthamiana* leaves. Total protein was extracted from *N. benthamiana* leaves expressing the indicated proteins at the designated time points. BiP proteins were analysed by immunoblot analysis using an anti-BiP antibody.

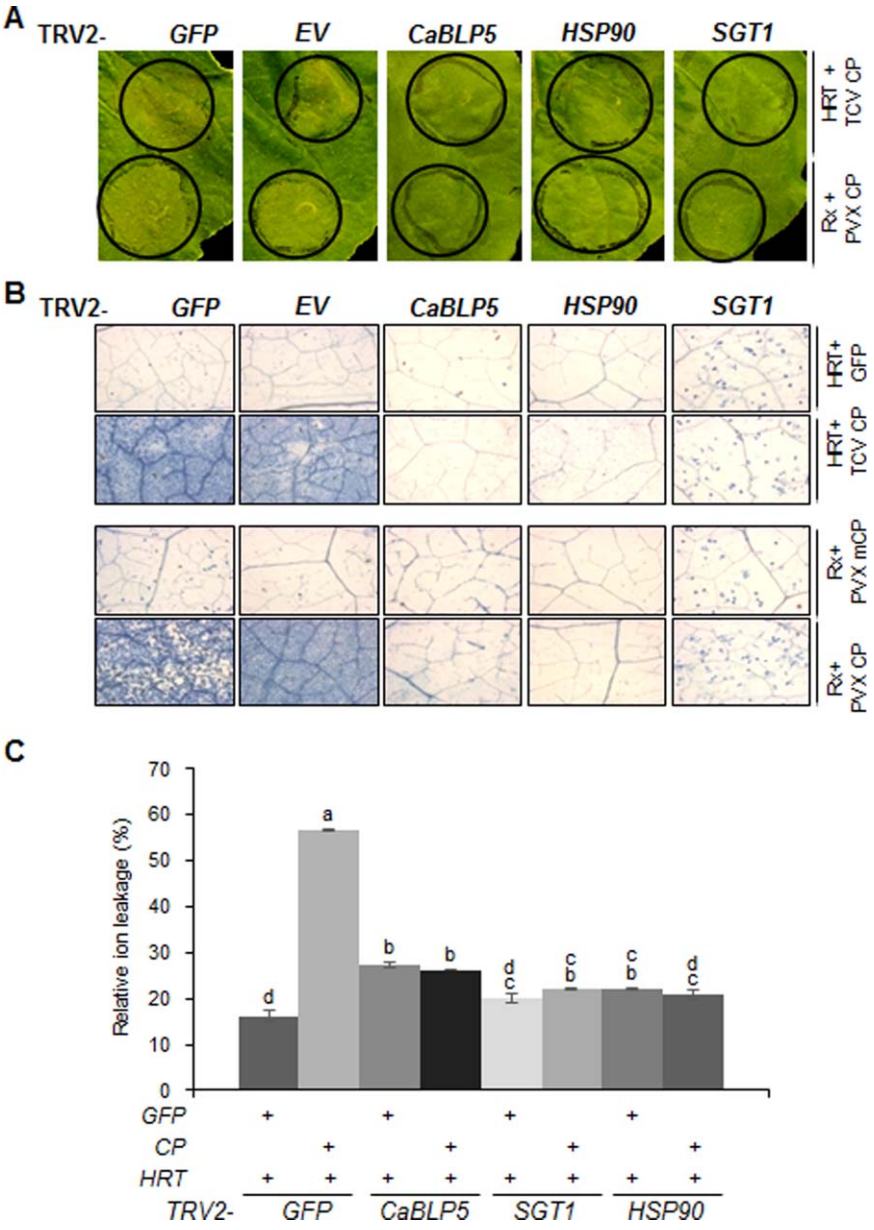

Fig. 4 Silencing of *CaBLP5* reduces the HRT- or Rx-mediated hypersensitive response (HR) to a level similar to that observed after suppression of *HSP90* and *SGT1*. (A) Visual HR phenotypes of the gene-silenced plants after agroinfiltration to co-express *Turnip crinkle virus* coat protein (TCV CP) and HRT, or *Potato virus X* (PVX) CP and Rx, a resistance protein. Black circles indicate the agroinfiltrated regions. Photographs were taken at 3 days post-infiltration (dpi). (B) Trypan blue staining at 40 h post-infiltration (hpi). Green fluorescent protein (GFP) and mutant type CP of PVX were employed as negative reference to TCV CP and PVX CP, respectively. After staining, the leaves were viewed under a light microscope to reveal necrotic plant cells. (C) Electrolyte leakage assay. Ion conductivity was measured at the indicated time points (hpi), and means ± standard deviation were calculated from 60 leaf discs (diameter, 1 cm) per construct; similar results were obtained in three independent experiments, and means with the same letter are not significantly different (*t*-test, $P < 0.05$).

and TRV2-empty vector (EV) leaves (Fig. 4A). Reduced HR was confirmed by trypan blue staining and electrolyte leakage assay (Fig. 4B,C). Co-infiltrated *HRT*/*GFP*, mutant *PVX CP* (*PVX mCP*; Bazzini *et al.*, 2006) and *Rx* were used as HR-negative controls. Similar to the results for HRT/TCV CP, Rx and PVX-CP co-expression also resulted in higher levels of BiP accumulation than those observed from Rx and PVX-mCP co-expression (Fig. S6, see Supporting Information). These results indicate that, in addition to *SGT1* and *HSP90* silencing, *BiP4/5* silencing disrupts HRT- and Rx-mediated HR in *N. benthamiana*.

To confirm the gene-specific down-regulation of *BiP4/5*, *HSP90* and *SGT1* in plants subjected to VIGS, qRT-PCR analyses were performed (Fig. 5A). Consistent with earlier results, *HSP90* and *SGT1* silencing affected the expression of each other, and the expression of *BiP4/5* was down-regulated by *SGT1* or *HSP90* silencing. However, *HSP90* expression was not affected in the *CaBLP5*-silenced plant, whereas *BiP4/5* and *SGT1* expression was strongly reduced. These results indicate that there is cross-regulation among the three genes, although the interaction of *BiP4/5* with *SGT1* and *HSP90* appears to differ. Expression of these genes in non-silenced *N. benthamiana* leaves was observed as a reference.

BiP5 is probably a chaperone. Therefore, we assessed whether BiP affects the accumulation of HRT protein. To determine the effect of BiP on the accumulation of HRT protein, we transiently expressed HA-tagged HRT protein together with TCV CP in *BiP4/5*-silenced *N. benthamiana* plants. We examined BiP protein

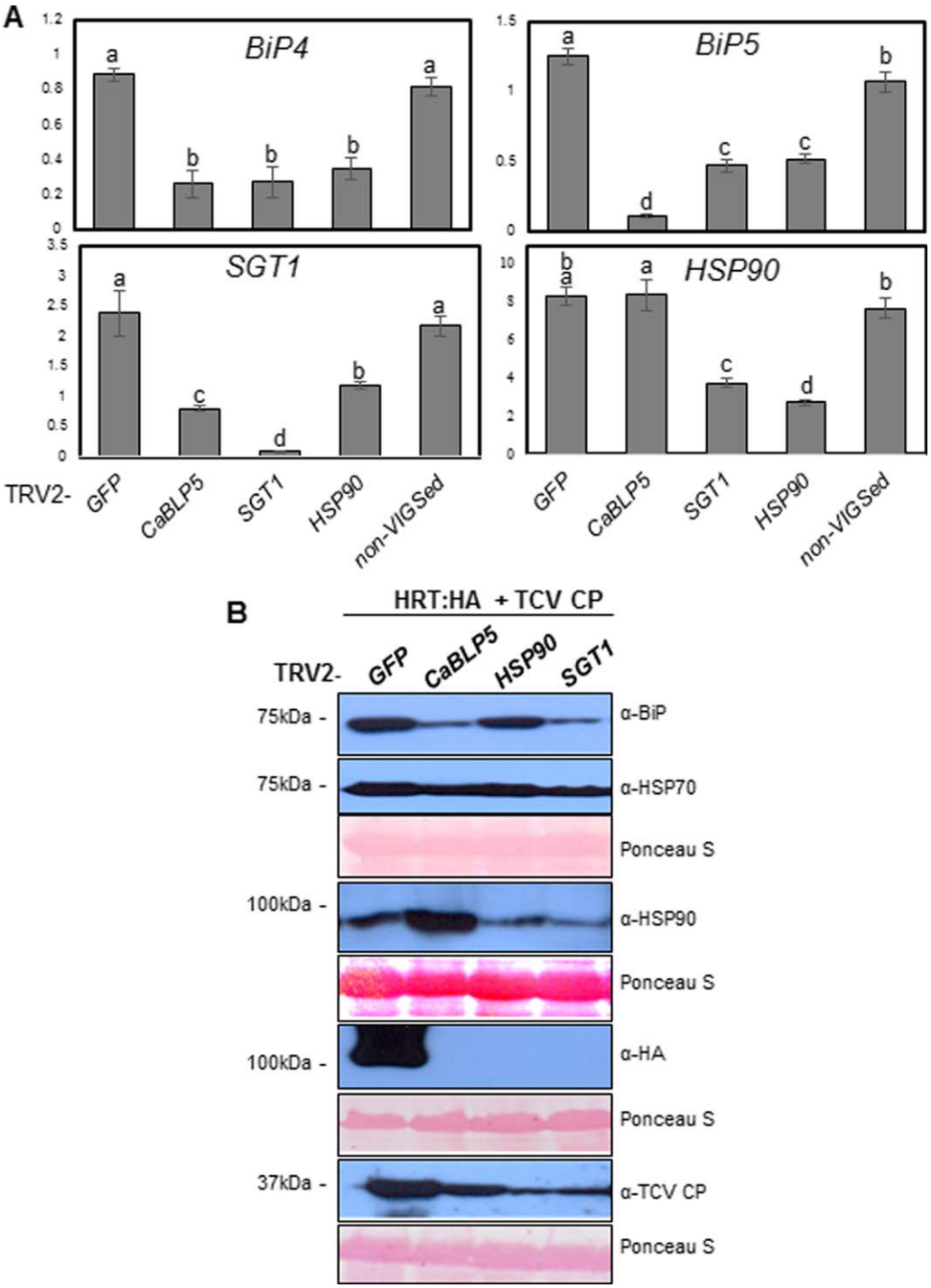

Fig. 5 Silencing of *BiP4/5*, *HSP90* or *SGT1* in *Nicotiana benthamiana* reduced HRT protein expression. (A) Verification of *CaBLP5*, *SGT1* and *HSP90* gene silencing in *N. benthamiana* plants. The expression of *CaBLP5*, *SGT1* and HSP90 genes in the silenced plants at 21 days post-infiltration (dpi) was analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the specific primers shown in Table S2, and the values were normalized to the level of *NbActin*. Error bars represent standard deviation ($n = 3$). Means with the same letter are not significantly different (*t*-test, $P < 0.05$). Normal *N. benthamiana* leaves (non-virus-induced gene silenced) were also used as a control. (B) Levels of BiP, HRT-HA, TCV CP and HSP90 in *BiP4/5*-silenced *N. benthamiana* leaves. Total proteins were isolated from leaves of the indicated gene-silenced *N. benthamiana*, co-infiltrated with haemagglutinin (HA)-tagged HRT and *Turnip crinkle virus* coat protein (TCV CP). The protein extracts were analysed by immunoblotting with anti-BiP, anti-cytosol-resident HSP70, anti-HSP90, anti-HA or anti-TCV CP antibodies to detect the protein levels.

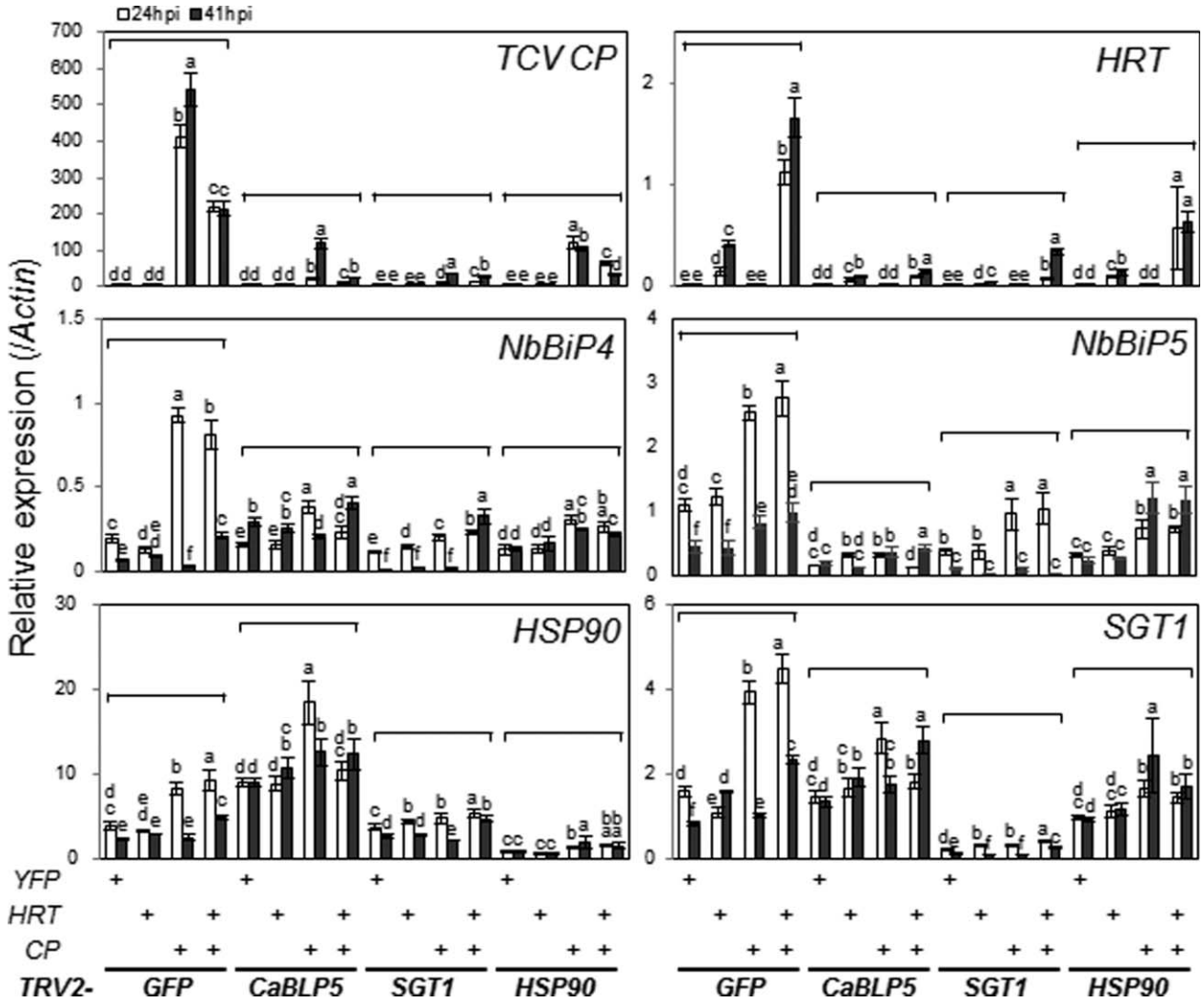

Fig. 6 HRT and *Turnip crinkle virus* coat protein (TCV CP) were impeded at mRNA levels in *BiP4/5*-silenced *Nicotiana benthamiana*. Total RNA was obtained from leaves of the gene-silenced plants after expression of either HRT alone or in conjunction with TCV CP at 24 h post-infiltration (hpi) (white bar) or 41 hpi (dark grey bar). The transcriptional levels of the specific genes were assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Error bars indicate standard deviation ($n = 3$). Bars with the same letter are not significantly different (*t*-test, $P < 0.05$). Two additional biologically independent experiments yielded similar results.

accumulation in the silenced plants during co-expression of HRT and TCV CP. Silencing of *BiP4/5*, *SGT1* and *HSP90* reduced BiP levels, whereas cytosolic HSP70 levels were not affected (Fig. 5). HSP90 accumulated at high levels in *BiP4/5*-silenced plants, whereas HSP90 levels were reduced in *SGT1*-silenced plants. These results raise the possibility that BiP, HSP90 and SGT1 functionally interact with different mechanisms during HRT-mediated HR. Next, we measured HRT protein levels in gene-silenced plant leaves using an anti-HA antibody. Although HRT proteins were efficiently expressed in TRV-*GFP* leaves, these proteins were not detected in *TRV2-CaBLP5*, *TRV2-HSP90* and *TRV2-SGT1* leaves (Fig. 5B). Previous studies have shown that HSP90 and SGT1 regulate the stability/accumulation of various R proteins (Holt *et al.*, 2005; Lu *et al.*, 2003), which raises the possibility that they serve as co-chaperone and chaperone for the assembly of an active R protein complex. Consistent with these previous reports, our results indicate that BiP4/5, HSP90 and SGT1 are important for HRT protein accumulation in *N. benthamiana*. TCV CP was detected in all silenced plants, although its accumulation was reduced in *CaBLP5*-, *HSP90*- and *SGT1*-silenced leaves.

BiP4/5, SGT1 and *HSP90* modulate *HRT* expression

To study *HRT* transcriptional regulation and the effects of *BiP4/5*, *HSP90* and *SGT1* silencing on HRT-mediated HR, we investigated the expression of *BiP4/5*, *HSP90* and *SGT1* in response to

HRT and/or TCV CP (Fig. 6). We did not observe any cell death response in HRT/TCV CP co-infiltrated leaves up to 24 h post-infiltration (hpi); however, after 36–40 hpi, HR appeared to be initiated specifically on leaf spots in which HRT and TCV CP were co-expressed. To analyse the gene expression patterns before and after HR was triggered, transcript levels were measured by qRT-PCR at 24 or 41 hpi (Fig. 6). In the same samples, we also confirmed the expression of *HRT* and *TCV CP* by qRT-PCR (Fig. 6).

First, we checked whether there were differences in gene expression in non-silenced, TRV-infected control leaves expressing *HRT* or *TCV CP* alone, and in leaves expressing *HRT* plus *TCV CP* (Fig. 6). We found significant differences in gene expression in response to *TCV CP* between *GFP*-silenced control and non-silenced *N. benthamiana* plants (Figs 3A and 6). In *GFP*-silenced control plants, transient *TCV CP* expression induced higher expression of *BiP4/5*, *SGT1* and *HSP90* than in *YFP*-expressing leaves (Fig. 6), although the *TCV CP* expression intensity and pattern were similar to those of non-silenced plants. Significant induction of *BiP4/5*, *SGT1*, *HSP90* and *HRT* was observed during HRT/TCV CP-mediated HR in *GFP*-silenced leaves. TCV CP-mediated induction of *BiP4/5*, *SGT1* and *HSP90* was observed only in the 24-hpi sample, whereas induction of these genes by HRT plus TCV CP was sustained until 41 hpi compared with the duration in the *YFP*-expressing control.

In TRV2-*GFP* plant leaves, *HRT* expression increased more than two-fold during HRT-mediated HR compared with the absence of HR (Fig. 6). However, *HRT* expression was most severely reduced in TRV2-*CaBLP5* leaves than in *GFP*-silenced control, *SGT1*- and *HSP90*-VIGSed leaves (Fig. 6). *HRT* expression in *SGT1*- and *HSP90*-silenced leaves showed a similar pattern to that in *GFP*-silenced leaves, although the overall expression was much less (Fig. 6). These results indicate that BiP4/5, HSP90 and SGT1 affect *HRT* expression at both the mRNA and protein levels (Figs 5B and 6). TCV CP expression was also strongly reduced in the *BiP4/5*-, *SGT1*- and *HSP90*-silenced plants, but the expression level did not differ significantly.

SGT1, BiP and HSP90 have been reported to be involved in *Agrobacterium*-mediated gene transfer in *N. benthamiana* (Anand and Mysore, 2013; Park *et al.*, 2014b); therefore, it is possible that low *HRT* expression in the silenced plants was caused by the low efficiency of transgene expression. To test this possibility, we searched for a gene that has similar function to that of *BiP*, *HSP90* or *SGT1*, but does not affect HRT/TCV CP-mediated HR. We found the *RAR1* gene (Anand and Mysore, 2013), which functions as a chaperone for several *R* genes involved in *Agrobacterium*-mediated gene transfer, but not in HRT-mediated immune responses in *Arabidopsis* (Chandra-Shekara *et al.*, 2004). We observed strong HR by co-expression of HRT/TCV CP in *RAR1*-silenced plants, although the HRT expression level was lower than that in the control (Fig. S8, see Supporting Information). These results suggest that the efficiency of *Agrobacterium*-mediated gene transfer is not the primary reason for HR suppression in *BiP4/5*-, *SGT1*- and *HSP90*-silenced plants.

Among the *BiP4/5*, S*GT1* and *HSP90* genes, *BiP4/5* silencing affected the expression of *HSP90* and *SGT1*. The induction of *HSP90* transcripts in response to TCV CP still occurred in *BiP4/5*-silenced plants relative to *YFP*-expressing leaves, whereas the induction during HRT-mediated HR was defective (Fig. 6). *SGT1* induction in response to both TCV CP and HRT/TCV CP was suppressed in *BiP4/5*-silenced plants. *SGT1* silencing reduced basal *BiP4/5* expression and abolished its induction by TCV CP and HRT/TCV CP; however, *HSP90* expression only affected its induction by TCV CP and HRT/TCV CP in *SGT1*-silenced plants. In *HSP90*-silenced plants, *SGT1* and *BiP4/5* expression was slightly reduced, and their induction in response to TCV CP and HRT/TCV CP was strongly suppressed. Collectively, these results suggest that co-chaperone *SGT1* expression might be correlated with both the expression of *BiP4/5* and *HSP90* chaperones with different interactions, and *BiP4/5* and *HSP90* expression might be regulated independently, especially in HRT-mediated immune responses.

BiP4/5 silencing alters the expression of stress-related genes

We determined whether *BiP4/5*, *SGT1* or *HSP90* changes the expression of defence-related and ER stress-responsive genes in response to TCV CP and/or HRT. We assessed the expression of two HR marker genes [*Hsr203J* and *HIN1* (harpin-induced 1)], four basal defence-related genes (*PR1a*, *PR2*, *CYP71D20* and *WRKY8*) and six ER stress-responsive genes [*Beclin 1*, *Calreticulin 1*, *PDI* (*protein disulfide isomerase*), $p58^{IPK}$, *bZiP60* and *PR4*] (Fig. 7). In cells transiently overexpressing *YFP*, selected ER stress-responsive genes were up-regulated in *CaBLP5*- and *HSP90*-silenced plants relative to *GFP*-silenced control plants (Fig. 7A). *Beclin1*, *PDI* and *PR4* were up-regulated in *HSP90*-silenced plants, whereas the expression of selected ER stress-responsive genes (except for *PR4*) in *SGT1*-silenced plants was comparable with that in *GFP*-silenced control plants (Fig. 7A). *PR4* is down-regulated by IRE1-dependent mRNA decay under ER stress (Mishiba *et al.*, 2013). Expression of *TCV CP* with or without *HRT* significantly up-regulated the examined ER stress-responsive genes, with the exception of *bZiP60* (Fig. 7A). Expression of *bZIP60* only increased in response to HRT and TCV CP co-expression. Expression of ER stress-responsive genes during HRT/TCV CP-triggered HR was significantly reduced in response to *BiP4/5* silencing, except for *PDI*, whereas induction of the same genes by *TCV CP* was not suppressed (Fig. 7A). By contrast, *SGT1* silencing induced the genes with the same expression patterns, but with lower intensities. *HSP90* silencing abolished the induction of all tested genes in response to TCV CP and

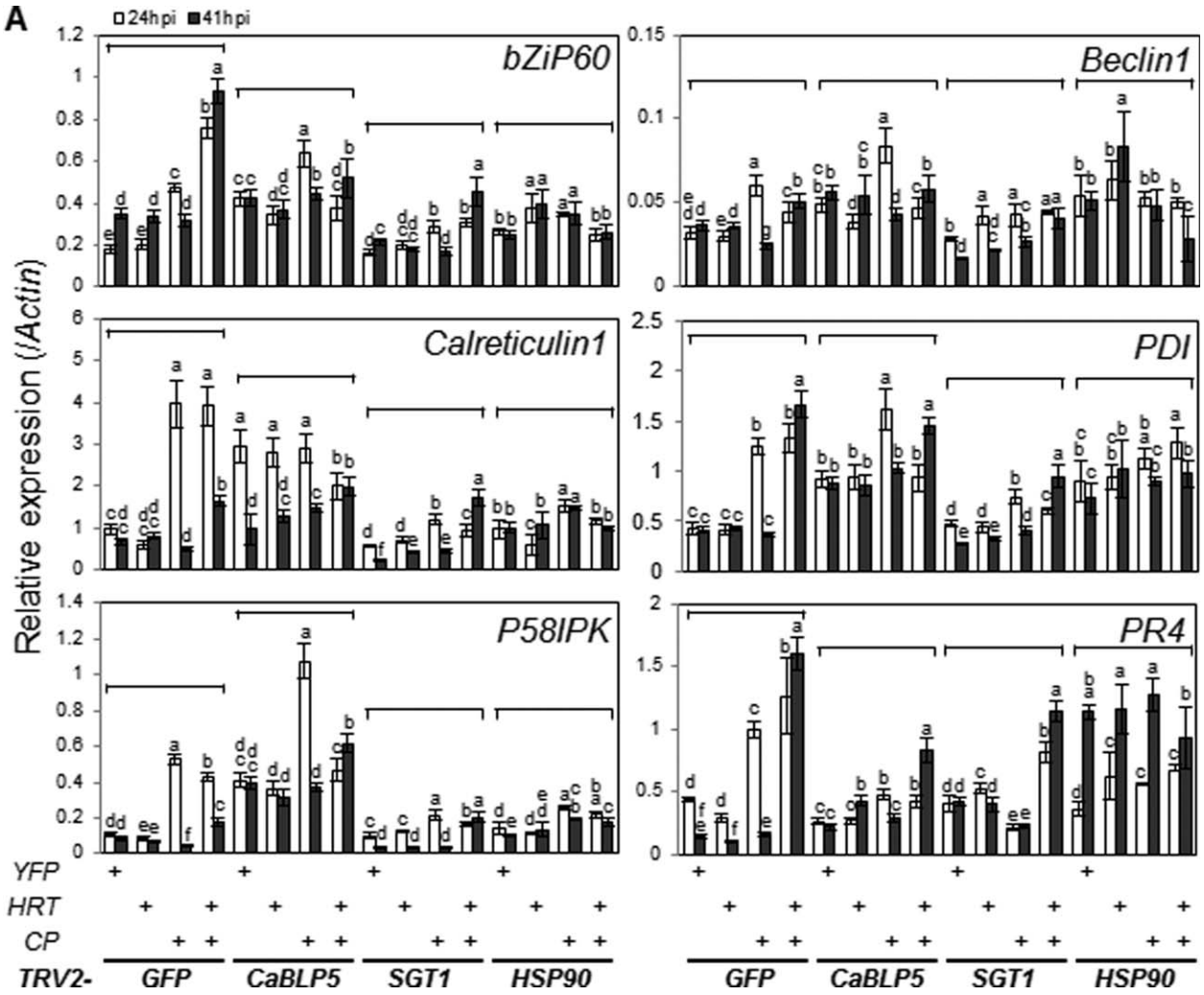

Fig. 7 Silencing of *BiP4/5* reduced defence or endoplasmic reticulum (ER) stress-related gene expression in *Nicotiana benthamiana*. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was conducted as shown in Fig. 6, using primers specific to ER stress-responsive genes (A) and defence-related genes (B). Bars with the same letter are not significantly different (*t*-test, $P < 0.05$). Two additional biologically independent experiments yielded similar results.

HRT/TCV CP. These combined results suggest that BiP4/5, HSP90 and SGT1 are involved in HRT-mediated HR via regulation of HRT, but have distinct relationships with ER stress responses.

Previous work has shown that *PR1a*, *HIN1* and *Hsr203J* are strongly induced during HR cell death (Heath, 2000). *HRT* and *TCV CP* co-expression induced the expression of the HR marker genes *PR1a*, *HIN1* and *HSR203*, and the basal defence genes *CYP71D20* and *WRKY8*, but *BiP4/5* silencing reduced the induction of all genes (Fig. 7B). The expression of most defence-related genes was similarly reduced in *HSP90*- and *SGT1*-silenced plants, with the exceptions of *PR1a* and *PR2* in *SGT1*-silenced plants, and *WRKY8* and *CYP71D20* in *HSP90*-silenced plants (Fig. 7B). Taken together, these results indicate that silencing of *BiP4/5*, *SGT1* and HSP90 triggers distinct ER stress responses and alters the expression patterns of defence-related genes in response to TCV CP or HRT/TCV CP.

Tm treatment blocks HRT-mediated HR in *N. benthamiana*

In mammals, BiPs are master regulators of signal transduction in the unfolded protein response (UPR) following ER stress (Zhang and Kaufman, 2004). In general, the accumulation of unfolded proteins in the ER triggers the UPR pathway, which results in elevated chaperone protein expression to maintain organellar homeostasis. Little is known about the effects of the UPR pathway on R-mediated HR. Therefore, we examined the effect of ER stress on R-mediated HR in *N. benthamiana*. We induced ER stress using

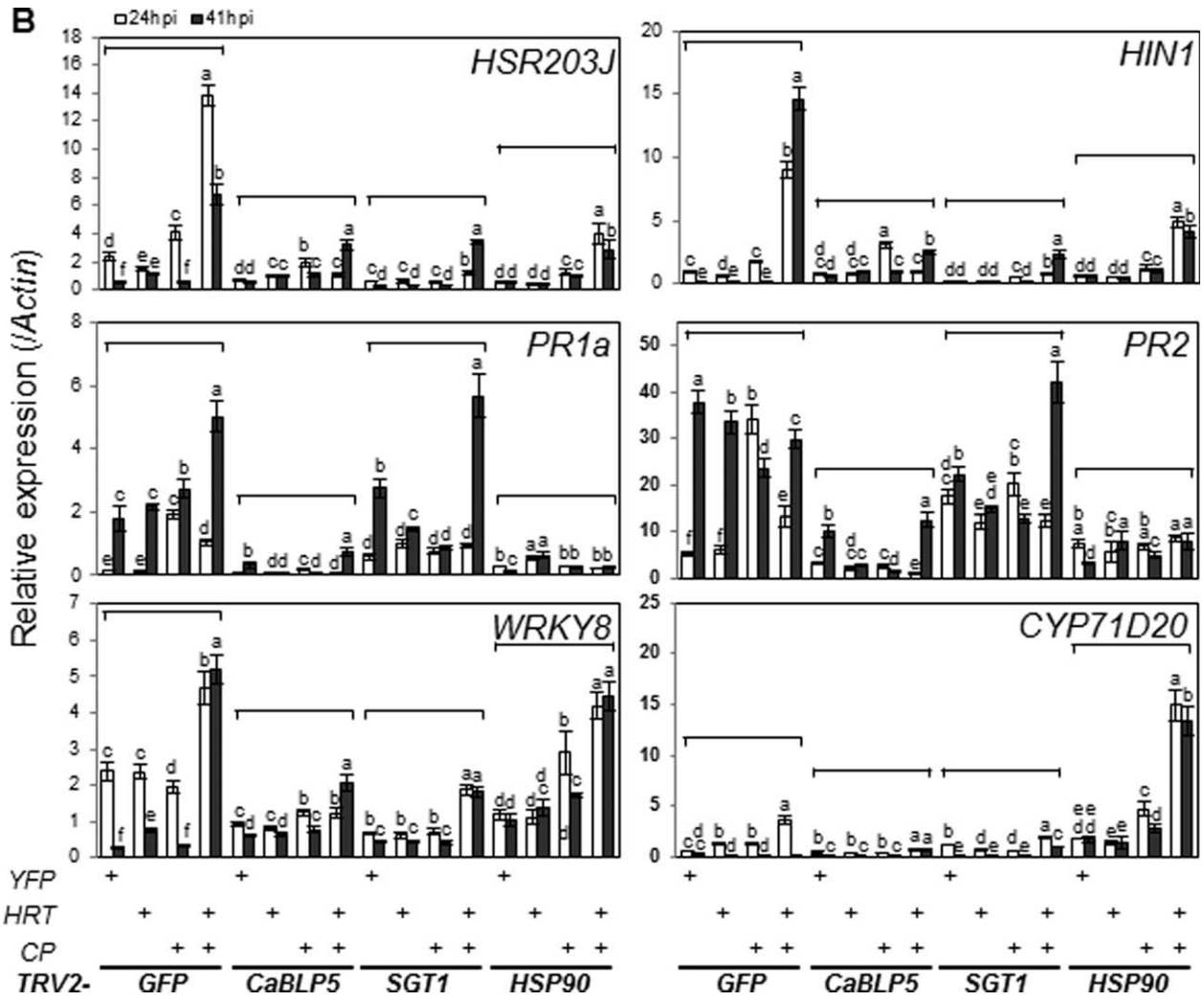

Fig. 7 Continued.

Tm, which inhibits *N*-glycosylation of proteins passing through the ER (Samali *et al.*, 2010) and activates the UPR pathway in mammals and plants. *Nicotiana benthamiana* leaves were co-infiltrated with HRT/TCV CP in the presence or absence of Tm. During cell death, plant cells accumulate fluorescent phenolic compounds in the cell wall, and the secondary metabolites of these compounds can be visualized by observing the fluorescence they emit under ultraviolet (UV) irradiation (Tang *et al.*, 1999). Treatment with Tm strongly inhibited HRT-mediated HR (Figs 8A and S9, see Supporting Information). Tm treatment increased BiP protein levels, as expected, but reduced HRT levels (Fig. 8B). These results indicate that HRT accumulation and stability are affected by ER stress and *N*-glycosylation, and dysregulation reduces cell death induction.

To confirm that Tm treatment triggered the UPR pathway, we measured *BiP5* and *bZiP60* expression by qRT-PCR, and monitored the BiP expression in *GFP*-overexpressing control plants. Expression of both genes increased as a function of Tm concentration (Fig. S10A, see Supporting Information), and BiP proteins accumulated in response to Tm treatment (Fig. S10B), confirming that Tm treatment triggers the UPR pathway. Cell death-like damage was observed in Tm-treated leaves at 5 dpi (data not shown).

DISCUSSION

Many mammalian viruses target ER stress sensors and utilize the host UPR pathway to enhance the folding of viral proteins or to modulate immune responses. The significance of ER stress and the UPR pathway in plant–virus interactions has started to be appreciated (Ye *et al.*, 2011, 2012; Zhang and Wang, 2012). However, it remains unclear how ER stress and the UPR pathway contribute to R protein-mediated HR. In this study, our VIGS screen for signalling factors that modulate HRT-mediated HR identified the ER-resident HSP70s, BiP4 and BiP5 proteins. We demonstrated that the BiP4/5-associated ER stress response has an important role in R protein-

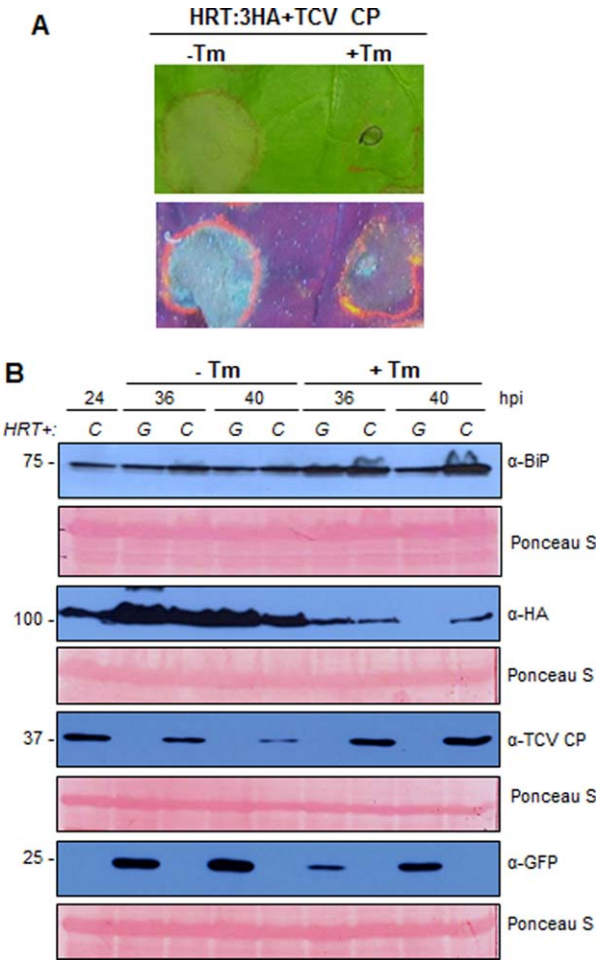

Fig. 8 Tunicamycin (Tm) treatment blocked the HRT-mediated hypersensitive response (HR) and HRT accumulation, but increased endoplasmic reticulum (ER)-resident heat shock protein 70 (HSP70) levels. (A) Verification of HR initiation after Tm treatment. HRT was co-expressed with *Turnip crinkle virus* coat protein (TCV CP) in leaves of *Nicotiana benthamiana* and Tm was infiltrated at a concentration of 10 μM. The images of the infiltrated leaves were photographed under white light or UV illumination at 3 days post-infiltration (dpi). (B) Effects of Tm treatment on the accumulation of the indicated proteins. Total protein was obtained at 24, 36 and 40 h post-infiltration (hpi) after expression of haemagglutinin (HA)-tagged HRT with TCV CP (CP) (C) or green fluorescent protein (GFP) (G). Western blotting was performed with anti-BiP, anti-HA, anti-TCV CP or anti-GFP antibodies to detect the protein levels.

mediated cell death, but that this response is dissimilar to the ER stress response induced by the *N*-glycosylation inhibitor Tm.

Recent studies have reported that plant viral proteins interact with components of the UPR machinery to enhance pathogenesis. For example, a movement protein of PVX, TGB3, resides in the ER during PVX infection (Ye *et al.*, 2011). This viral protein induces the expression of ER-resident chaperones, including BiP, through the bZiP60 signalling pathway (Garcia-Marcos *et al.*, 2009; Ye *et al.*, 2011). Silencing of *bZiP60* suppresses PVX replication, and BiP overexpression inhibits the TGB3-induced cell death response (Ye *et al.*, 2012). Unlike TGB3, TCV CP did not induce the expression of any ER stress-responsive genes; however, TCV CP overexpression induced several ER stress-responsive genes in TRV-*GFP*-silenced control plants (Fig. 7A). TRV is a positive-strand RNA virus with a bipartite genome. TRV can move systemically in many plants (Ratcliff *et al.*, 2001). Several studies have identified a cysteine-rich 16K protein of TRV as the main suppressor of RNA silencing (Martínez-Priego *et al.*, 2008; Reavy *et al.*, 2004), which occurs as the result of the inhibition of the formation of the initial RNA silencing complex by interference with AGO (argonaute) proteins (Fernández-Calvino *et al.*, 2016). Previous work has reported the strong RNA-silencing activity of TCV CP in *N. benthamiana* using an *Agrobacterium*-mediated transient expression study (Qu *et al.*, 2003). Therefore, the transient overexpression of TCV CP increased TRV accumulation probably via the inhibition of RNA silencing-mediated plant basal defence responses, leading to the induction of ER stress responses and suggesting that this response may be proportional to the level of the virus. A few reports have explained the involvement of ER stress responses in plant immunity. The tobacco *N* gene, which confers resistance against *Tobacco mosaic virus* (TMV), interacts with multiple cytosolic and ER-resident quality control (QC) components that are essential for *N*-mediated HR and TMV resistance (Caplan *et al.*, 2009). In cucumber, it has been reported that chemically induced UPR promotes SAR (Sticher and Metraux, 2000). Nagashima *et al.* (2014) have shown that exogenous SA treatment activates bZIP28, and consequently induces UPR gene expression in an IRE1-dependent and NPR1-independent manner. Chandra-Shekara *et al.* (2004) have reported that a high SA level up-regulates *HRT* transcription and HRT-mediated resistance is required for SA-dependent signal transduction (Chandra-Shekara *et al.*, 2004). The current study showed diminished *HRT* expression in *BiP4/5*-, *SGT1*- and *HSP90*-silenced leaves, and HRT/TCV CP-specific bZIP60 induction. These results suggest that *HRT* expression is up-regulated by ER stress response-triggered transcription factors, such as bZIP60. Further analysis will be performed to explore this possibility by investigating *HRT* expression in ER stress-related genetic mutants.

The HRT/TCV CP-induced HR was disrupted in both *BiP4/5*-silenced and Tm-treated plants. The ER-resident BiP protein levels responded in opposite ways to these two conditions, as follows: BiP protein levels were greatly reduced in *BiP4/5*-silenced plants, whereas BiP proteins accumulated in Tm-treated plants (Figs 5B and 8B). In both cases, ER-QC was dysfunctional, which probably significantly reduced HRT protein accumulation. ER-QC is crucial in plants for the abundance, quality and signalling of transmembrane immune receptors, including receptor-like R proteins, such as Cf4 (Liebrand *et al.*, 2012) and Ve1 (Liebrand *et al.*, 2014), and pathogen-associated molecular pattern receptors, such as EFR (elongation factor Tu receptor) and, to a lesser degree, FLS2 (flagellin-sensitive 2)

(Nekrasov *et al.*, 2009). There are four *BiP* and three *calreticulin* genes in tomato. Among these, only *calreticulin 3a* silencing disrupted Cf4 protein function, whereas silencing of any *BiP* (except for *BiP4*) or *calreticulin* genes impaired Ve1-mediated resistance (Liebrand *et al.*, 2012). This indicates a differential requirement for ER-QC in these two *R* gene-mediated responses. Phylogenetic analysis revealed that *NbBiP5* is very similar to *NbBiP4* (Figs S2 and S4). Therefore, we compared *NbBiP4* and *NbBiP5* expression patterns, and found that they were largely similar (Fig. 3). Plant BiPs exhibit tissue-specific expression and differential regulation in response to abiotic and biotic stresses through distinct signalling pathways (Moreno and Orellana, 2011; Tintor and Saijo, 2014). These lines of evidence suggest that different mechanisms can regulate the expression of even very close homologues, such as BiP4 and BiP5, in *N. benthamiana*. To investigate this issue further, we are currently engaged in a study to determine the specific requirement of ER-QC components for HRT, such as BiP4, BiP5 and CRTs, using a specific VIGS approach to target individual ER-QC components and yeast two-hybrid experiments.

Although many studies have used VIGS to study plant–pathogen interactions, its use has several limitations (Senthil-Kumar and Mysore, 2011). For instance, in studies designed to identify plant genes involved in antiviral mechanisms, silencing of the candidate plant gene can affect viral replication, thus making it hard to determine whether the phenotype is caused by the silencing of the plant gene or high virus proliferation (Burch-Smith *et al.*, 2004). To study further the biological significance of *BiP4/5* in HRT-mediated TCV disease resistance, we are generating transgenic *N. benthamiana* plants expressing *HRT* and studying the role of BiPs in *Arabidopsis* TCV resistance. Because TRV and TCV viruses have different properties (e.g. in replication strategy, proliferation, viral RNA levels and silencing suppressor), the effect of TRV-mediated VIGS must be carefully evaluated to arrive at a full understanding of TCV resistance in *N. benthamiana*.

EXPERIMENTAL PROCEDURES

Plant materials

The GFP-expressing *N. benthamiana* line used for gene silencing experiments was kindly provided by Dr Dinesh Kumar (University of California-Davis, CA, USA). For protein expression studies, leaves of 4-week-old *N. benthamiana* plants were subjected to agroinfiltration (*Agrobacterium*-mediated transient transfection). Plants were grown in pots at 23°C in a growth chamber under a 16 h light/8 h dark cycle.

Plasmid construction for VIGS

To clone the *N. benthamiana* cDNA fragments corresponding to the *HSP90* gene, we searched GenBank sequence information and designed specific primers. A 542-bp cDNA fragment of *N. benthamiana HSP90* (AY368904) was amplified using a pair of primers (5′-TGACTGGGAGGAGCATTTG-3′ and 5′-GGTACCGCAGCAGTTCAGC-3′). This cDNA was cloned into the T&A vector (Bioneer, Daejeon, South Korea). The cDNA sequence was confirmed by DNA sequencing, and it was cloned into the *pTRV2* silencing vector.

VIGS and *Agrobacterium*-mediated transient assays

VIGS and *Agrobacterium*-mediated transient assays were performed according to the protocol described previously (Lee *et al.*, 2013; Liu *et al.*, 2002b) Each silencing experiment was repeated at least three times, and each experiment included at least three independent plants.

For transient expression assay, *A. tumefaciens* strain GV2260 containing the *HRT*, *TCV CP*, *GFP*, *Rx*, *PVX-mCP*, *PVX-CP* or *HRT:3HA* gene, driven by the 35S promoter, was cultured overnight and harvested by centrifugation. Cells were resuspended in infiltration medium [10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.6, 10 mM $MgCl_2$ and 200 μM acetosyringone] to an optical density at 600 nm (OD_{600}) of 0.5 for *HRT:3HA* and 0.1 for the other genes, and then incubated for 2 h at room temperature. The inoculum was infiltrated into 4-week-old *N. benthamiana* or 3-week-old gene-silenced *N. benthamiana* leaves with a 1-mL needleless syringe. To observe the effect of Tm (Calbiochem, Los Angeles, CA, USA) on HR or protein status, drugs were prepared as 10 mM stocks in dimethylsulfoxide (DMSO) and stored at −20°C until use.

Cell death measurement by trypan blue staining or electrolyte leakage assay

Cell death was monitored by trypan blue staining according to a previously described method (Koch and Slusarenko, 1990). Samples were analysed with an Axiophot photomicroscope (Zeiss, Munich, Germany) under bright-field optics.

The extent of cell death was measured quantitatively by monitoring electrolyte leakage. Ten leaf discs (diameter, 1 cm) were punched out of the infiltrated leaf tissue, and washed in distilled water for 30 min with gentle shaking to remove ions released by sampling-related injury. Then, leaf discs were transferred to a plate containing 10 mL of distilled/deionized water, which was incubated in a growth chamber until 70 hpi. Conductivity measurements were taken at the indicated times using a NeoMet EC Meter EC-470L conductivity meter (iSTE, Seoul, South Korea); three replicates were performed for each treatment.

qRT-PCR analysis

Total RNAs were extracted from infiltrated leaf tissues using Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and cDNA synthesis was performed with MMLV reverse transcriptase (Invitrogen, Grand Island, NY, USA) using oligo(dT), according to the manufacturer's instructions. qRT-PCR was performed as described by Liu *et al.* (2002b) with some modifications. Briefly, the PCR conditions were as follows: first denaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 20 s; annealing at a primer-specific temperature for 30 s; elongation at 72°C for 20 s; and a melting curve consisting of 1°C steps from 65 to 95°C. Reactions were performed on a CFX Connect™ Real-Time System (Bio-Rad, Hercules, CA, USA). Relative expression levels of the indicated genes, normalized against the expression level of *NbActin*, were calculated via the comparative C(T) method. The primers used are listed in Table S2 (see Supporting Information).

Statistical analysis

Data were analysed using an analysis of variance (ANOVA) to evaluate the expression levels of the relative genes or the conductivity of ion leakage. Student's *t*-test, or one-way ANOVA, followed by Tukey's honestly significant difference (HSD) test, was used to compare the means of the treatments in each experiment. All statistical analyses were conducted using SPSS v. 18.

Immunoblot analysis

Total cellular protein was extracted from three infiltrated leaf discs using urea lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0), and the extracts were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a poly(vinylidene difluoride) (PVDF) membrane (Pierce, Rockford, IL, USA) using a wet-transfer apparatus. The protein blots were incubated overnight in 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, pH 7.4) supplemented with 0.1% (v/v) Tween 20 (PBST) containing anti-HA (Roche, Lewes, UK), anti-TCV CP (Kang *et al.*, 2010), anti-BiP (SPA818), anti-HSP70 (SPA817) (Stressgen Biotechnology, Victoria, BC, Canada), anti-HSP90 and anti-GFP (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies.

ACKNOWLEDGEMENTS

We thank Dr Dinesh Kumar for providing the pTRV1, pTRV2 and pTRV2:*SGT1* constructs, Dr Daniel F. Klessig for providing the HA-tagged *HRT* clone and antisera against TCV CP, and Dr David Baulcombe for providing the *Rx*, *PVX-mCP* and *PVX-CP* clones. This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2009-0090805), and by the KRIBB initiative program to JMP. The research was partly funded by the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009762), Rural Development Administration to C-SO and the Texas State University-Faculty Standup Program to H-GK.

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **95**, 10 306–10 311.

Anand, A. and Mysore, K.S. (2013) The role of RAR1 in Agrobacterium-mediated plant transformation. *Plant Signal. Behav.* **8**, e26784. doi: 10.4161/psb 26784.

Bazzini, A.A., Asurmendi, S., Hopp, H.E. and Beachy, R.N. (2006) Tobacco mosaic virus (TMV) and potato virus X (PVX) coat proteins confer heterologous interference to PVX and TMV infection, respectively. *J. Gen. Virol.* **87**, 1005–1012.

Bendahmane, A., Farnham, G., Moffett, P. and Baulcombe, D.C. (2002) Constitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the Rx locus of potato. *Plant J.* **32**, 195–204.

Bhattarai, K.K., Li, Q., Liu, Y., Dinesh-Kumar, S.P. and Kaloshian, I. (2007) The MI-1-mediated pest resistance requires Hsp90 and Sgt1. *Plant Physiol*, **144**, 312–323.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B. and Dinesh-Kumar, S.P. (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J.* **39**, 734–746.

Caplan, J.L., Zhu, X., Mamillapalli, P., Marathe, R., Anandalakshmi, R. and Dinesh-Kumar, S.P. (2009) Induced ER chaperones regulate a receptor-like kinase to mediate antiviral innate immune response in plants. *Cell Host Microbe*, **6**, 457–469.

Carvalho, H.H., Silva, P.A., Mendes, G.C., Brustolini, O.J., Pimenta, M.R., Gouveia, B.C., Valente, M.A., Ramos, H.J., Soares-Ramos, J.R. and Fontes, E.P. (2014) The endoplasmic reticulum binding protein BiP displays dual function in modulating cell death events. *Plant Physiol.* **164**, 654–670.

Chandra-Shekara, A.C., Navarre, D., Kachroo, A., Kang, H.G., Klessig, D. and Kachroo, P. (2004) Signaling requirements and role of salicylic acid in HRT- and rrt-mediated resistance to turnip crinkle virus in Arabidopsis. *Plant J.* **40**, 647–659.

Collier, S.M. and Moffett, P. (2009) NB-LRRs work a "bait and switch" on pathogens. *Trends Plant Sci.* **14**, 521–529.

Cooley, M.B., Pathirana, S., Wu, H.J., Kachroo, P. and Klessig, D.F. (2000) Members of the Arabidopsis HRT/RPP8 family of resistance genes confer resistance to both viral and oomycete pathogens. *Plant Cell*, **12**, 663–676.

Denecke, J., Goldman, M.H., Demolder, J., Seurinck, J. and Botterman, J. (1991) The tobacco luminal binding protein is encoded by a multigene family. *Plant Cell*, **3**, 1025–1035.

Fernández-Calvino, L., Martínez-Priego, L., Szabo, E.Z., Guzmán-Benito, I., González, I., Canto, T., Lakatos, L. and Llave, C. (2016) Tobacco rattle virus 16K silencing suppressor binds ARGONAUTE 4 and inhibits formation of RNA silencing complexes. *J. Gen. Virol.* **97**, 246–257.

Garcia-Marcos, A., Pacheco, R., Martianez, J., Gonzalez-Jara, P., Diaz-Ruiz, J.R. and Tenllado, F. (2009) Transcriptional changes and oxidative stress associated with the synergistic interaction between Potato virus X and Potato virus Y and their relationship with symptom expression. *Mol. Plant–Microbe Interact.* **22**, 1431–1444.

Gassmann, W. and Bhattacharjee, S. (2012) Effector-triggered immunity signaling: from gene-for-gene pathways to protein–protein interaction networks. *Mol. Plant–Microbe Interact.* **25**, 862–868.

Hammond-Kosack, K.E. and Parker, J.E. (2003) Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. *Curr. Opin. Biotechnol.* **14**, 177–193.

Heath, M.C. (2000) Hypersensitive response-related death. *Plant Mol. Biol.* **44**, 321–334.

Holt, B.F. III, Belkhadir, Y. and Dangl, J.L. (2005) Antagonistic control of disease resistance protein stability in the plant immune system. *Science*, **309**, 929–932.

Hubert, D.A., Tornero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu, K. and Dangl, J.L. (2003) Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. *EMBO J.* **22**, 5679–5689.

Hubert, D.A., He, Y., McNulty, B.C., Tornero, P. and Dangl, J.L. (2009) Specific Arabidopsis HSP90.2 alleles recapitulate RAR1 cochaperone function in plant NB-LRR disease resistance protein regulation. *Proc. Natl. Acad. Sci. USA*, **106**, 9556–9563.

Kalinski, A., Rowley, D.L., Loer, D.S., Foley, C., Buta, G. and Herman, E.M. (1995) Binding-protein expression is subject to temporal, developmental and stress-induced regulation in terminally differentiated soybean organs. *Planta*, **195**, 611–621.

Kang, H.G., Kuhl, J.C., Kachroo, P. and Klessig, D.F. (2008) CRT1, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to turnip crinkle virus. *Cell Host Microbe*, **3**, 48–57.

Kang, H.G., Oh, C.S., Sato, M., Katagiri, F., Glazebrook, J., Takahashi, H., Kachroo, P., Martin, G.B. and Klessig, D.F. (2010) Endosome-associated CRT1 functions early in resistance gene-mediated defense signaling in Arabidopsis and tobacco. *Plant Cell*, **22**, 918–936.

Ko, C.B., Woo, Y.M., Lee, D.J., Lee, M.C. and Kim, C.S. (2007) Enhanced tolerance to heat stress in transgenic plants expressing the GASA4 gene. *Plant Physiol Biochem.* **45**, 722–728.

Koch, E. and Slusarenko, A. (1990) Arabidopsis is susceptible to infection by a downy mildew fungus. *Plant Cell*, **2**, 437–445.

Kohno, K., Normington, K., Sambrook, J., Gething, M.J. and Mori, K. (1993) The promoter region of the yeast KAR2 (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol. Cell. Biol.* **13**, 877–890.

Leborgne-Castel, N., Jelitto-Van Dooren, E.P., Crofts, A.J. and Denecke, J. (1999) Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress. *Plant Cell*, **11**, 459–470.

Lee, A.S. (1992) Mammalian stress response: induction of the glucose-regulated protein family. *Curr. Opin. Cell Biol.* **4**, 267–273.

Lee, J.H., Kim, Y.C., Choi, D. and Park, J.M. (2013) Identification of novel pepper genes involved in Bax- or INF1-mediated cell death responses by high-throughput virus-induced gene silencing. *Int. J. Mol. Sci.* **14**, 22 782–22 795.

Liebrand, T.W., Smit, P., Abd-El-Haliem, A., de Jonge, R., Cordewener, J.H., America, A.H., Sklenar, J., Jones, A.M., Robatzek, S., Thomma, B.P., Tameling, W.I. and Joosten, M.H. (2012) Endoplasmic reticulum-quality control

chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato. *Plant Physiol.* **159**, 1819–1833.
Liebrand, T.W., Kombrink, A., Zhang, Z., Sklenar, J., Jones, A.M., Robatzek, S., Thomma, B.P. and Joosten, M.H. (2014) Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to *Verticillium*. *Mol. Plant Pathol.* **15**, 109–117.
Lin, B.L., Wang, J.S., Liu, H.C., Chen, R.W., Meyer, Y., Barakat, A. and Delseny, M. (2001) Genomic analysis of the Hsp70 superfamily in *Arabidopsis thaliana*. *Cell Stress Chaperones*, **6**, 201–208.
Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. (2002a) Virus-induced gene silencing in tomato. *Plant J.* **31**, 777–786.
Liu, Y., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. (2002b) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* **30**, 415–429.
Lu, R., Malcuit, I., Moffett, P., Ruiz, M.T., Peart, J., Wu, A.J., Rathjen, J.P., Bendahmane, A., Day, L. and Baulcombe, D.C. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J.* **22**, 5690–5699.
Martínez-Priego, L., Donaire, L., Barajas, D. and Llave, C. (2008) Silencing suppressor activity of the Tobacco rattle virus-encoded 16-kDa protein and interference with endogenous small RNA-guided regulatory pathways. *Virology*, **376**, 346–356.
Mishiba, K., Nagashima, Y., Suzuki, E., Hayashi, N., Ogata, Y., Shimada, Y. and Koizumi, N. (2013) Defects in IRE1 enhance cell death and fail to degrade mRNAs encoding secretory pathway proteins in the Arabidopsis unfolded protein response. *Proc. Natl. Acad. Sci. USA*, **110**, 5713–5718.
Moreno, A.A. and Orellana, A. (2011) The physiological role of the unfolded protein response in plants. *Biol. Res.* **44**, 75–80.
Nagashima, Y., Iwata, Y., Ashida, M., Mishiba, K. and Koizumi, N. (2014) Exogenous salicylic acid activates two signaling arms of the unfolded protein response in Arabidopsis. *Plant Cell Physiol.* **55**, 1772–1778.
Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z.H., Lacombe, S., Rougon, A., Bittel, P., Kiss-Papp, M., Chinchilla, D., van Esse, H.P., Jorda, L., Schwessinger, B., Nicaise, V., Thomma, B.P., Molina, A., Jones, J.D. and Zipfel, C. (2009) Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J.* **28**, 3428–3438.
Noël, L.D., Cagna, G., Stuttmann, J., Wirthmüller, L., Betsuyaku, S., Witte, C.P., Bhat, R., Pochon, N., Colby, T. and Parker, J.E. (2007) Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. *Plant Cell*, **19**, 4061–4076.
Oh, C.S., Pedley, K.F. and Martin, G.B. (2010) Tomato 14-3-3 protein 7 positively regulates immunity-associated programmed cell death by enhancing protein abundance and signaling ability of MAPKKK {alpha}. *Plant Cell*, **22**, 260–272.
Park, C.J., Bart, R., Chern, M., Canlas, P.E., Bai, W. and Ronald, P.C. (2010) Overexpression of the endoplasmic reticulum chaperone BiP3 regulates XA21-mediated innate immunity in rice. *PLoS One*, **5**, e9262.
Park, C.J., Song, M.Y., Kim, C.Y., Jeon, J.S. and Ronald, P.C. (2014a) Rice BiP3 regulates immunity mediated by the PRRs XA3 and XA21 but not immunity mediated by the NB-LRR protein, Pi5. *Biochem. Biophys. Res. Commun.* **448**, 70–75.
Park, S.Y., Yin, X., Duan, K., Gelvin, S.B. and Zhang, Z.J. (2014b) Heat shock protein 90.1 plays a role in Agrobacterium-mediated plant transformation. *Mol. Plant*, **7**, 1793–1796.
Qu, F., Ren, T. and Morris, T.J. (2003) The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. *J. Virol.* **77**, 511–522.
Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* **25**, 237–245.
Reavy, B., Dawson, S., Canto, T. and MacFarlane, S.A. (2004) Heterologous expression of plant virus genes that suppress post-transcriptional gene silencing results in suppression of RNA interference in Drosophila cells. *BMC Biotechnol.* **4**, 1–9.
Samali, A., Fitzgerald, U., Deegan, S. and Gupta, S. (2010) Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. *Int. J. Cell Biol.* **2010**, p. 830307.
Senthil-Kumar, M. and Mysore, K.S. (2011) New dimensions for VIGS in plant functional genomics. *Trends Plant Sci.* **16**, 656–665.
Shirasu, K. (2009) The HSP90-SGT1 chaperone complex for NLR immune sensors. *Annu. Rev. Plant Biol.* **60**, 139–164.
Sticher, L.A. and Metraux, J.P. (2000) Inhibitors of N-linked glycosylation induce systemic acquired resistance in cucumber. *Physiol. Mol. Plant Pathol.* **56**, 245–252.
Tamagnone, L., Merida, A., Stacey, N., Plaskitt, K., Parr, A., Chang, C.F., Lynn, D., Dow, J.M., Roberts, K. and Martin, C. (1998) Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. *Plant Cell*, **10**, 1801–1816.
Tang, X., Xie, M., Kim, Y.J., Zhou, J., Klessig, D.F. and Martin, G.B. (1999) Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell*, **11**, 15–30.
Tintor, N. and Saijo, Y. (2014) ER-mediated control for abundance, quality, and signaling of transmembrane immune receptors in plants. *Front. Plant Sci.* **5**, 65.
Wang, D., Weaver, N.D., Kesarwani, M. and Dong, X. (2005) Induction of protein secretory pathway is required for systemic acquired resistance. *Science*, **308**, 1036–1040.
Wrobel, R.L., O'Brian, G.R. and Boston, R.S. (1997) Comparative analysis of BiP gene expression in maize endosperm. *Gene*, **204**, 105–113.
Xu, G., Li, S., Xie, K., Zhang, Q., Wang, Y., Tang, Y., Liu, D., Hong, Y., He, C. and Liu, Y. (2012) Plant ERD2-like proteins function as endoplasmic reticulum luminal protein receptors and participate in programmed cell death during innate immunity. *Plant J.* **72**, 57–69.
Ye, C., Dickman, M.B., Whitham, S.A., Payton, M. and Verchot, J. (2011) The unfolded protein response is triggered by a plant viral movement protein. *Plant Physiol.* **156**, 741–755.
Ye, C.M., Kelly, V., Payton, M., Dickman, M.B. and Verchot, J. (2012) SGT1 is induced by the potato virus X TGBp3 and enhances virus accumulation in *Nicotiana benthamiana*. *Mol. Plant*, **5**, 1151–1153.
Zhang, K. and Kaufman, R.J. (2004) Signaling the unfolded protein response from the endoplasmic reticulum. *J. Biol. Chem.* **279**, 25 935–25 938.
Zhang, L. and Wang, A. (2012) Virus-induced ER stress and the unfolded protein response. *Front. Plant Sci.* **3**, 293.
Zhao, Y., DelGrosso, L., Yigit, E., Dempsey, D.M.A., Klessig, D.F. and Wobbe, K.K. (2000) The amino terminus of the coat protein of Turnip crinkle virus is the AVR factor recognized by resistant Arabidopsis. *Mol. Plant–Microbe Interact.* **13**, 1015–1018.
Zimmermann, R., Eyrisch, S., Ahmad, M. and Helms, V. (2011) Protein translocation across the ER membrane. *Biochim. Biophys. Acta*, **1808**, 912–924.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Haemagglutinin (HA)-tagged HRT is functional. Co-expression of *HRT-HA* and *Turnip crinkle virus* (*TCV*) coat protein (CP) induced the hypersensitive response (HR) in *Nicotiana benthamiana*. Numbers indicate concentration (optical density at 600 nm, OD_{600}) of *Agrobacterium* cells. The images were photographed at 3 days post-infiltration (dpi).

Fig. S2 Phylogenetic analysis of CaBLP5 (GenBank accession no. KC912859) and its homologues. The amino acid sequences of 26 CaBLP5 homologues were imported into MEGA 6 (Tamura *et al.*, 2013) for multiple sequence alignment with ClustalW (Larkin *et al.*, 2007). Phylogenetic analysis was performed using the neighbour-joining (Saitou and Nei., 1987) and bootstrap methods. The bootstrap consensus tree was inferred from 500 replicates. The scale bar indicates the lengths of the branches (relative evolutionary distance). The protein sequences are deposited in GenBank under the following accession numbers: *Solanum lycopersicum* SlBiP5 (XP_004234985.1) and SlBiP (NP_001234636.1), *Solanum tuberosum* StBiP5-L (XP_006350519.1) and StBiP-L (XP_006343810.1), *Nicotiana benthamiana* NbBLP4 (ACK55195.1), *Zea mays* ZmBiP3 (NP_001105894.1) and ZmBiP2 (NP_001105893.1), *Arabidopsis thaliana* AtBiP2 (NP_851119.1) and AtBiP1 (NP_198206.1), *Nicotiana tabacum* NtBiP4 (Q03684.1), *Cucumis sativus* CsBiP5-L

(XP_004143862.1), *Vitis vinifera* VvBiP5 (XP_002263323.1) and VvBiP5-L (XP_002276268.2), *Nicotiana sylvestris* NsBiP5 (XP_009773333.1), NsBiP4 (XP_009788736.1), NsBiP (XP_009802727.1) and NsBiP4-L (XP_009770477.1), *Nicotiana tomentosiformis* NtoBiP5 (XP_009592769.1), NtoBiP4 (XP_009588550.1), NtoBiP (XP_009593820.1) and NtoBiP4-L (XP_009619852.1), *Ricinus communis* RsHSP (XP_002518865.1), *Cucumis sativus* CsBiP5-l (XP_004143862.1) and *Glycine max* GmBiP (XP_003525327.2), GmBiP isoform A (NP_001234941.1) and GmBiP isoform B (NP_001238736.1).

Fig. S3 Multiple sequence alignment of CaBLP5 and six *Nicotiana benthamiana* BiP genes with CLUSTAL 2.1. Asterisks on the bottom line of the alignment indicate identical residues in a given sequence position. Within the aligned sequences, dashes indicate the gaps that were inserted to optimize the alignment. The *CaBLP5* nucleotide sequences in red were used for the *Tobacco rattle virus* (TRV)2-*CaBLP5* construct. Residues underlined in red represent gene-specific primer sequences used for the detection of gene expression in *GFP*- or *CaBLP5*-silenced plants. Residues in turquoise indicate stretches of more than 21 nucleotides (Liu *et al.*, 2002a) that are identical between TRV2-*CaBLP5* and the corresponding *NbBiP* homologues. It should be noted that TRV2-*CaBLP5* contains a stretch of 21 nucleotides with 100% identity to *Nbv3K645786225* and *Nbv3K645789686*, and multiple 21-nucleotide stretches with 100% identity to *Nbv3K685813373*, but no stretches of 21 nucleotides with perfect matches to other *BiP* homologues.

Fig. S4 Phylogenetic analysis of CaBLP5 and its tobacco and *Arabidopsis* homologues. The amino acid sequences of 11 CaBLP5 homologues were imported into MEGA 6 for multiple sequence alignment with CLUSTALW. Phylogenetic analysis was performed using the neighbour-joining and bootstrap methods. The bootstrap consensus tree was inferred from 500 replicates. The scale bar indicates the lengths of the branches (relative evolutionary distance). GenBank accession numbers for the homologous proteins are given in the legend of Fig. S2.

Fig. S5 Putative amino acid sequence alignment of CaBLP5 (AGS42239) and its *Nicotiana benthamiana* homologues. The amino acid sequences of three CaBLP5 homologues were aligned with CLUSTALW. Asterisks on the bottom line of the alignment indicate identical residues in a given sequence position; single and double dots refer to highly and moderately conserved residues, respectively. Within the aligned sequences, dashes indicate gaps that were inserted to optimize the alignment.

Fig. S6 Induction of Rx-mediated hypersensitive response (HR) resulted in an increase in the level of BiPs. Rx and mutant *Potato virus X* coat protein (PVX mCP) or wild-type PVX CP (PVX CP) were co-expressed in leaves of *Nicotiana benthamiana*. Total protein was extracted from *N. benthamiana* leaves expressing the indicated proteins at the designated time points and analysed by immunoblotting with anti-BiP or anti-cytosolic heat shock protein (HSP) 70 antibodies. Ponceau S staining of Rubisco was used as a loading control.

Fig. S7 Morphological phenotype of the gene-silenced *Nicotiana benthamiana* plant. *GFP*- (*green fluorescent protein*) and *PDS* (*phytoene desaturase*)-silenced plants were used as negative and positive controls for the virus-induced gene silencing (VIGS) experiment. The silenced plants were photographed at 21 days post-infiltration (dpi).

Fig. S8 Silencing of *RAR1*. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to assess the efficiency of gene silencing. Primers directed to a specific gene or *NbActin* were used with equal amounts of cDNA from silenced plants. Numbers indicate PCR cycles. (B) HRT/coat protein (CP)-mediated hypersensitive response (HR) induction in the leaves of *RAR1*-silenced plants. (C) *HRT* transcriptional levels in *GFP*- and *RAR1*-silenced plants. The values were normalized to the expression of *NbActin*. Error bars represent standard deviation ($n = 3$). Means with the same letter are not significantly different (*t*-test, $P < 0.001$).

Fig. S9 Effect of tunicamycin (Tm) on cell death during co-expression of HRT/*Turnip crinkle virus* coat protein (TCV CP) or Rx/*Potato virus X* coat protein (PVX CP). TM, an endoplasmic reticulum (ER) stress-inducing chemical, was co-infiltrated at the indicated concentrations, and infiltration buffer was used as a control. Photographs were taken at 3 days post-infiltration (dpi).

Fig. S10 Triggering of the unfolded protein response (UPR) pathway by tunicamycin (Tm) treatment. (A) Expression of *BiP5* and *bZiP60* in *Tobacco rattle virus*2-*green fluorescent protein* (TRV2-*GFP*)-infected plants by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Error bars present the standard error of three replicates. Asterisks indicate a significant difference from the corresponding control (Student's *t*-test, $^{**}P < 0.005$). Tm 10, 10 μM Tm; Tm 50, 50 μM Tm. (B) Effects of Tm treatment on the BiP protein level. Total protein was prepared at the indicated time points (h). BiP and GFP proteins were detected using anti-BiP and anti-GFP antibodies.

Table S1. Putative *CaBLP5* homologues identified in *Nicotiana benthamiana*. *CaBLP5* homologues were isolated using BLAST searches in the *N. benthamiana*_transcriptome_v3_ unigenes95 database (http://benth-web-pro-1.ucc.usyd.edu.au/blast/search.php). Six C*aBLP5* homologues were retrieved, which have an e-value of less than 10^{-9} and more than 80% identity with positive orientation.

Table S2. List of the oligonucleotide primers used in this study.