Running Title: Plant viral MP is an elicitor of ER stress and UPR

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The Unfolded Protein Response is Triggered by a Plant Viral Movement Protein

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ABSTRACT

Potato virus X (PVX) infection in Nicotiana benthamiana plants leads to increased transcript levels of several stress related host genes including, bZIP60, SKP1, BiP, PDI, CRT, and CAM. bZIP60 is a key transcription factor that functions in response to endoplasmic reticulum (ER) stress and induces expression of ER resident chaperones (BiP, PDI, CRT, and CAM). SKP1 is a component of SCF (SKP1-Cullin-F box protein) ubiquitin ligase complexes that target proteins for proteasomal degradation. Expression of PVX TGBp3 from a heterologous vector induces the same set of genes in N. benthamiana and Arabidopsis leaves. Virus induced gene silencing was employed to knock down expression of bZIP60 and SKP1, and the numbers of infection foci on inoculated leaves was reduced and systemic virus accumulation was altered. Silencing bZIP60 led to suppression of BiP and SKP1 transcript levels suggesting that bZIP60 might be an upstream signal transducer. Over expression of TGBp3 led to localized necrosis, but co-expression of TGBp3 with BiP abrogated necrosis demonstrating that the UPR induction potentially alleviates ER stress related cell death. Steady state levels of PVX replicase and TGBp2 (which reside in the ER) proteins were unaltered by the presence of TGBp3, suggesting that TGBp3 does not contribute to their turnover. Taken together, PVX TGBp3 induced ER stress leads to upregulation of bZIP60 and UPR related gene expression which may be important to regulate cellular cytotoxicity that could otherwise lead to cell death if viral proteins reach high levels in the ER.
INTRODUCTION

Various cellular disturbances cause unfolded proteins to accumulate in the endoplasmic reticulum (ER) prompting a response that is conserved across kingdoms known as the unfolded protein response (UPR). External stimuli such as pathogen invasion, nutrient depletion, or glucose deprivation can exert stress on the ER by causing vigorous protein synthesis, aberrations in Ca\textsuperscript{2+} or redox regulation, inhibition of protein glycosylation, or protein transfer to the Golgi. These responses increase the levels of misfolded proteins in the ER and trigger the UPR. Export of malformed proteins from the ER into the cytosol is followed by degradation via the ubiquitin–proteasome pathway (Supplemental Fig. S1). Thus the purpose of the UPR is to restore normal ER function, relieve stress exerted on the ER, and prevent the cytotoxic impact of malformed proteins (Jelitto-Van Dooren et al., 1999; Xu et al., 2005; Slepak et al., 2007; Urade, 2007; Preston et al., 2009).

Many UPR signaling components are conserved among mammals, yeast, and plants although mammals and plants each have additional factors that lead to unique and complex sets of cellular responses (Xu et al., 2005; Zhang and Kaufman, 2006)(Supplemental Fig. S1). Nutrient depletion or pharmacological agents, such as tunicamycin, have been used to map the plant signaling pathways relating to ER stress and UPR (Williams and Lipkin, 2006). In both mammals and plants, the UPR mechanism involves increasing synthesis of several ER resident proteins needed to restore proper protein folding such as the ER luminal binding protein (\textit{BiP}), protein disulfide isomerase (\textit{PDI}), calreticulin (\textit{CRT}), and calmodulin (\textit{CAM}) (Supplemental Fig. S1) (Navazio et al., 2001; Ellgaard and Helenius, 2003; Oh et al., 2003; Urade, 2007; Seo et al., 2008). In tobacco, \textit{NtBLP-4} (the ER luminal binding protein \textit{BiP}), \textit{NtCRT}, and \textit{NtPDI} were specifically upregulated by ER stress inducing compounds (Denecke et al., 1991; Denecke et al., 1995; Iwata and Koizumi, 2005). In fact \textit{NtBLP-4} is linked to pro-survival responses in plants and its overexpression alleviates ER stress (Leborgne-Castel et al., 1999). Other plant pro-survival factors include SDF2 which is a target of the UPR and contributes to plant development (Schott et al., 2010).

In homeostatic mammalian cells, BiP binds to ER resident protein sensors such as IRE1, ATF6, and PERK (Supplemental Fig. S1). However, during ER stress, BiP binds to misfolded proteins and releases its hold on these sensors. At the same time, IRE1, ATF6, and PERK respond to ER stress by inducing pathways that typically upregulate cellular pro-survival signals
but under extreme conditions can lead to pro-death signals. IRE1 and ATF6 represent sensors in the pro-survival pathways. In plants, *AtIRE1* and *AtBAG7* bind BiP and contribute to the maintenance of UPR (Koizumi et al., 2001; Lu and Christopher, 2008; Williams et al., 2010).

Basic-region leucine zipper (bZIP) transcription factors are also fundamental contributors to UPR (Supplemental Fig. S1). In mammals and yeast, the relevant bZIP transcription factors are XBP1, ATF6, and Hac1. In *Arabidopsis*, *bZIP60* is a membrane bound transcription factor that is strongly induced following application of ER stress inducing chemicals such as tunicamycin (Martinez and Chrispeels, 2003). *bZIP60* is activated by intra membrane proteolysis (Supplemental Fig. S1) and is translocated into the nucleus where it upregulates expression of certain ER resident chaperones such as *BiP*, *PDI* and *CRT* (Iwata and Koizumi, 2005; Urade, 2007; Iwata et al., 2008; Lu and Christopher, 2008; Iwata et al., 2009). In tobacco, *NtbZIP60* also localizes to the ER, responds to chemically induced ER stress, and is activated by non-host bacterial pathogens (Tateda et al., 2008).

The SCF-type E3 ubiquitin ligase complex, containing SKP1 and Cullin1 (Murai-Takebe et al., 2004), contributes to the elimination of misfolded proteins in mammalian and plant cells via the 26S proteasome (Supplemental Fig. S1) (Wang et al., 2006). However, little is known about protein recruitment for proteasomal degradation as part of the plant UPR. Der1-like proteins from maize were reported to aid degradation of misfolded proteins (Kirst et al., 2005). SKP1 is a highly conserved core protein in the SCF complex. The *Arabidopsis* and *N. benthamiana* SKP1 proteins participate in host defense to polerovirus infection (Pazhouhandeh et al., 2006), response to jasmonates during defense (Xu et al., 2002; Ren et al., 2005; Gfeller et al., 2010), and in *Agrobacterium* tumorogenicity (Tzfira et al., 2004; Zaltsman et al., 2010). The role of ubiquitin ligase complexes in ER stress as well as in pathogen defense and susceptibility is particularly intriguing since this study investigates the role of ER stress in virus infection. Without knowing a specific link between UPR and the proteasome in plants, we chose to examine changes in *NbSKP1* expression following application of a viral ER stress elicitor in an attempt to link virus-induced UPR with at least one component of the SCF complex.

Many mammalian RNA viruses manipulate host UPR signaling pathways to promote viral RNA translation and persistence in infected cells. These events are necessary to manage the increase in protein translation resulting from virus infection and membrane expansion needed for replication and maturation (Yu et al., 2006). For example, flaviviruses such as *Japanese
encephalitis virus (JEV) and dengue viruses (DEN) trigger the IRE1-XBP1 pathway which leads to enhanced protein folding abilities, ER expansion, and up regulation of the secretory system (Urano et al., 2000). While viral modification of the ER architecture has been explored in plants, there are no reported studies examining the role for UPR pathways in plant viral disease. Therefore, we decided to investigate whether Potato virus X (PVX) can also modulate UPR signaling pathways to modify the cellular environment as described for many mammalian viruses. We explored the role of ER stress in PVX pathogenesis because of the broad range of viral proteins that are known to associate with the ER. For example, we recently reported that the PVX replicase associates with the ER and that PVX infection is accompanied by expansion of the ER network (Bamunusinghe et al., 2009). The PVX TGBp2 and TGBp3 are low molecular weight proteins (12 and 8 kDa) that also associate with the ER and contribute to cell-to-cell movement (Zamyatnin et al., 2002; Krishnamurthy et al., 2003; Mitra et al., 2003; Schepetilnikov et al., 2005; Zamyatnin et al., 2006). TGBp2 has two transmembrane domains while TGBp3 has a single N-terminal transmembrane domain. ER association is necessary for these two proteins to promote virus spread, although the exact role of the ER in virus egress from the cell is not yet elucidated. Importantly, preliminary investigations indicate that TGBp2 and TGBp3 have distinct interactions with the ER network. When expressed in the absence of PVX infection, TGBp2 induces novel granular vesicles to bud from the ER (Ju et al., 2005). TGBp3 on the other hand is distributed throughout the cortical ER network in membrane bound subdomains alongside the viral replicase, and it is also packaged into TGBp2-containing granular vesicles (Samuels et al., 2007). These TGBp2-granular vesicles are required for virus cell-to-cell movement although it is not known whether they function as containers carrying viral cargo to the periphery of the cell or play an alternative role in virus maturation and egress (Ju et al., 2005; Ju et al., 2007; Verchot-Lubicz et al., 2010).

Wild-type and aberrant forms of TGBp3 are exported from the ER and degraded by the 26S proteasome (Ju et al., 2005; Ju et al., 2008). Given the role of the proteasome in regulating viral protein accumulation and evidence that virus infection leads to expansion of the ER, we hypothesized that PVX infection could cause mild ER stress leading to upregulation of the UPR. In this study, we provide evidence that PVX TGBp3 upregulates UPR-related genes including bZIP60 when it is expressed from the PVX genome or heterologous expression vectors. We investigate the role of ER stress in maintaining persistent virus infection, and conclude that UPR
is a contributing factor toward promoting virus spread. We also link bZIP60 to SKP1 and UPR signaling and systemic accumulation of PVX.

RESULTS
Upregulation of UPR during PVX Infection

We compared the gene expression profiles obtained using Arabidopsis and potato microarrays, which were reported by Whitham et al., (2003) and Garcia-Marcos et al., (2009), to identify common ER stress regulated genes that are induced by PVX infection. In both investigations, UPR-related ER-resident chaperones as BiP, PDI, and CRT were upregulated, but only the potato microarray detected CAM (Supplemental Table S1). bZIP60, SKP1, but not IRE1 were predicted to be upregulated in the published potato cDNA microarray probed with samples taken from PVX-infected N. benthamiana leaves (Garcia-Marcos et al., 2009). Neither of the ER stress-related sensors bZIP60 nor IRE1 were represented on the Affymetrix Arabidopsis 8K GeneChip oligonucleotide microarray, and therefore, their expression was not determined (Supplemental Table S1). The expression of SKP1 was not reported to be altered in Arabidopsis, although it was represented on the microarray.

To further investigate gene expression associated with the UPR in PVX-infected leaves, qRT-PCR assessment of host transcript accumulation was performed using total RNA isolated from PVX-GFP infected N. benthamiana leaves at 3 and 9 days post inoculation (dpi). Green fluorescent foci appear on the inoculated leaves at 3 dpi. The plants were fully and systemically infected at 9 dpi, although we extracted RNA from the inoculated leaves. Zero dpi represents samples that were harvested just before plants were inoculated with PVX-GFP. Given that the genome sequence for N. benthamiana is incompletely annotated, primers were designed for qRT-PCR based on the sequences of homologs from N. tabacum (NtZIP60, NtBLP-4, NtCAM, NtCRT, and NtPDI) that have high homology to ESTs identified in the potato microarray (Supplemental Table S1).

Non-parametric analysis was used to describe the distribution of gene expression levels determined by qRT-PCR at 0, 3, or 9 dpi. This method of analysis provides excellent characterization of gene induction when using plant tissues that are not synchronously infected with PVX and/or if host gene expression is transiently altered (Bamunusinghe et al., 2009). Kruskal-Wallis tests (nonparametric analyses of variance) were performed to assess the
relationship of time on the various response variables. P-values associated with the tests of
equality of medians for each gene examined were less than 0.001, except for CAM whose p-
value was 0.014. All p-values indicate that PVX infection caused a significant increase in the
expression of each gene over time.

PVX infection leads to a general increase in population values (representing fold-changes
in gene expression) for bZIP60 (Fig. 1) at 3 and 9 dpi. The median values at 9 dpi are 3 to 4-fold
higher, the border of the box representing the upper 75th percentile reaches 4 to 5-fold increase,
and there is a maximum increase of 10-fold among outliers (Fig. 1; p <0.0001). Such a general
increase allows us to conclude that the gene is induced.

We can also conclude that a gene is induced based on the box plot analysis whereby values
have a positively skewed distribution. The median values for SKP1, BiP, PDI, CRT, and CAM at
0 dpi were approximately 0.9 with the range of values extending from 0.05 to 2.3 (Fig. 1). The
median values for BiP and SKP1 increased to 3.4 fold at 9 dpi. The range of values for BiP and
SKP1 expression are positively skewed (represented by elongation of box and whiskers above
the median) and showed elevated values of 5.6- and 7.5-fold, respectively, and maximum values
of 8- and 10-fold (Fig. 1; p < 0.001).

PVX infection also leads to significant changes in CRT transcript accumulation, while
CAM and PDI values show a mild positive change at 9 dpi. The boxes and whiskers for PDI,
CRT, and CAM were generally small indicating low dispersion of values among the plants
analyzed. For CAM, PDI, and CRT, the median values at 9 dpi increased to approximately 1.7,
2.0, and 2.5-fold respectively. CRT expression among plants in the 75th percentile showed up to
4.5- fold increase (p = 0.007) while for CAM and PDI the value ranges were moderately changed
(Fig. 1; PDI, p < 0.01; CAM, p = 0.0143). The increased expression of bZIP60, BiP, CAM, PDI,
and SKP1 clearly suggests that PVX infection coincides with the upregulation of UPR.

TGBp3 Causes UPR-related Gene Induction Following Agro-delivery

To study the mechanism of UPR induction and identify the viral inducers we employed
Agrobacterium-mediated transient expression in a reproducible and quantitative assay. The
entire PVX genome and each PVX gene was expressed from a binary vector containing the
Cauliflower mosaic virus (CaMV) 35S promoter (Fig 2A) for agro-delivery to N. benthamiana
leaves. By comparing host gene expression following ago-delivery of each PVX gene we could
learn whether UPR induction is a general response to virus infection or is specifically induced by a single PVX factor. PVX encodes three proteins that associate with the ER: replicase (Rep), TGBp2, and TGBp3 and any or all of these could cause upregulation of ER stress related genes. Given that UPR is often associated with ER stress, we predicted that one or more ER-resident factors would be responsible for host gene induction.

Initial immunoblot analysis was carried out to confirm PVX gene expression in *N. benthamiana* leaves following agro-delivery. Since we lack antisera detecting PVX replicase (Rep), TGBp2, or TGBp3 either a myc- or 6x-His tag was fused to these PVX genes (Fig 2A) and immunoblot analysis was carried out using either anti-myc or penta-His antisera. We introduced the 6x-His tag into the PVX genome at the 3’ end of TGBp3 without altering virus infectivity but we were not able to make similar insertions to fuse myc tags to Rep or TGBp2. The 3’ end of Rep overlaps with the TGBp1 subgenomic promoter and adding a tag here would destroy the function of the subgenomic promoter. Also the TGBp2 promoter and coding sequence overlaps with TGBp1 and TGBp3 and a fusion in the endogenous sequence would eliminate the functions of the overlapping genes. However, the N-terminal myc fusion was expected to be functional, because GFP-TGBp2 fusions facilitated PVX infection (Ju et al., 2005). Thus immunoblot analysis in Fig 2B confirms Rep and TGBp2 expression from the CaMV 35S promoter but cannot compare the levels of expression of the same genes from the PVX genome. On the other hand the levels of TGBp3, TGBp1, and coat protein (CP) are comparable when expressed from the PVX genome or directly from the CaMV35S promoter (Fig 2B).

*N. benthamiana* leaves were infiltrated with each binary construct, total RNA was extracted at 2 and 5 d post infiltration and qRT-PCR was carried out. Controls include leaves infiltrated with buffer (mock) or *A. tumefaciens* alone (Agro). Since agro-infiltration results in synchronous delivery of PVX and each PVX gene to plant cells, the values obtained were less dispersed than in plants inoculated with purified virus.

Interestingly, TGBp3-delivery resulted in 3.5- to 4-fold higher levels of *BiP*, *bZIP60*, *CRT*, and *SKP1* transcript accumulation at 2 dpi in comparison to mock inoculated plants. At 5 dpi, *BiP*, *bZIP60*, *CRT*, *CAM*, and *SKP1* showed 4- to 5-fold higher expression (Fig 2C p<0.05). *PDI* induction was approximately 2-fold. Similarly the expression levels of *bZIP60*, *BiP*, *CAM*, *PDI*, and *SKP1* in PVX- infected *N. benthamiana* leaves averaged 2 to 4-fold above the mock
control at 2 d post infiltration (Fig. 2C; p ≤ 0.1). Since the level of TGBp3 expression is comparable with the level of expression from the PVX genome, it is not likely that such high levels of \( bZIP60 \), \( BiP \), \( CAM \), \( PDI \), and \( SKP1 \) are due to cytotoxic overexpression of TGBp3, but is more likely due to a real effect of TGBp3 on the host. Since the level of gene induction during PVX infection is not as profound as TGBp3 alone, it is reasonable to consider that there might be other viral proteins interacting with TGBp3 during virus infection which may suppress the effect of TGBp3 on the host.

We also notice 2-fold induction of \( BiP \) and \( bZIP60 \) by Rep and CP at either 2 or 5 dpi. TGBp2 also induces SKP1 although suggesting that its upregulation may be independent of \( bZIP60 \) controlled pathways. The effects of these other PVX proteins are not as profound as TGBp3. Notably \( CRT \) appears to be induced by PVX and several of its genes suggesting that its induction is more likely the result of a generalized response.

**UPR induction in Arabidopsis**

Given the microarray data identified the same set of host factors were induced in Arabidopsis and \( N. benthamiana \) plants, we employed the same TGBp3-containing binary vector to examine the ability of TGBp3 to induce UPR-related genes in Arabidopsis. Immunoblot analysis also confirmed successful expression of TGBp3His from the CaMV 35S promoter following agro-delivery to Arabidopsis leaves (Fig 3A). For Arabidopsis, the average level of induction of \( AtbZIP60 \), \( AtBiP2 \) and \( AtCAM2 \) was 2 to 5.5-fold at 2 and 5 d post infiltration, indicating these are early and stable responses to the viral protein (Fig. 3A; p < 0.05). \( AtCRT2 \) and \( AtPDI2-1 \) were upregulated at 5 d post infiltration (Fig. 3A; p < 0.05). \( AtSKP1 \), \( AtCRT2 \), and \( AtPDI2 \) transcripts accumulated to significant levels ranging from 2 to 5.5-fold above control samples at 5 d post infiltration (Fig. 3A; p < 0.05).

**Induction is related to the strength of the promoter driving TGBp3 expression**

We also examined whether gene induction in \( N. benthamiana \) is dependent upon the promoter driving expression or the concentration of \( A. tumefaciens \) infiltrated into the leaves. Leaves were infiltrated with \( A. tumefaciens \) carrying binary plasmids containing TGBp3 fused either to the NOS or CaMV 35S promoter. Protein expression was lower from the NOS promoter relative to the CaMV 35S promoter (Fig 3B). Various dilutions of \( A. tumefaciens \)
(OD$_{600}$=1.0, 0.1, 0.01) were delivered to *N. benthamiana* leaves to determine if there is a dosage dependent response. In general, host gene induction was greatest at 2 and 5 dpi when 1.0 OD$_{600}$ of *A. tumefaciens* solution was used and induction was proportionally less with each dilution (Fig 3B). The NOS promoter is weaker than the CaMV35S promoter and this led to somewhat lower fold changes in expression of UPR-related genes (Fig. 3B). Collectively, these data demonstrate that expression of TGBp3 alone is sufficient to induce expression of UPR-related genes and that the TGBp3 levels correlate with the magnitude of induction.

**Suppression of bZIP60 and Its Impact on BIP and SKP1 Expression**

BiP is an ER resident member of the Hsp70 family and its expression is a marker for ER stress and the UPR. bZIP60 is known to upregulate BiP as part of an ER stress response (Iwata and Koizumi, 2005) but it is not the only transcription factor responsible for its upregulation. SKP1 is a component of the SCF-type E3 ubiquitin ligase complex (Murai-Takebe et al., 2004) that is implicated in the elimination of misfolded proteins in mammalian and plant cells via the 26S proteasome (Supplemental Fig. S1) (Wang et al., 2006) and it is unknown whether bZIP60 might also be responsible for its upregulation. Because *bZIP60*, *BiP*, and *SKP1* expression was induced by TGBp3, we hypothesized that *bZIP60* is an upstream transducer responsible for elevated levels of *BiP* and possibly *SKP1*. Importantly, Fig. 2 also shows that there is a 2-fold induction of BiP by other PVX factors and approximately 3-fold induction of SKP1 by TGBp2 at 5 dpi and therefore it is possible that both genes are only partially under the control of *bZIP60*. Therefore, silencing *bZIP60* was expected to suppress *BiP* and *SKP1* mRNA.

We employed *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) to knock down expression of *bZIP60*. A 600 bp fragment of *NbbZIP60* was cloned into the TRV vector (Ratcliff et al., 2001; Dong et al., 2007). *N. benthamiana* plants at the four leaf stage were pre-treated with buffer, TRV1 plus TRV2 empty vector, or TRV1 plus TRV2-bZIP60 or TRV1 plus TRV2-SKP1. RNA was harvested from upper leaves of silenced plants 14 days later, and then semi-quantitative RT-PCR was carried out to examine the expression of *bZIP60*, *BiP*, and *SKP1* (Fig. 4). As expected, plants that were pre-treated buffer or TRV alone showed similar levels of *bZIP60*, *BiP*, or *SKP1*. In plants treated with TRV2-bZIP60, bZIP60 was suppressed by 77% below mock treated plants. In plants treated with TRV2-SKP1, SKP1 levels were suppressed 60% below mock treated plants. We examined *BIP* and *SKP1* expression in *bZIP60*
suppressed plants and found expression of these genes was also suppressed 72% and 65%, respectively below mock treated plants. While we cannot assume that expression of these genes are solely driven bZIP60, these data shows that knocking down bZIP60 severely hampers BiP and SKP1 expression. Thus, BiP and SKP1 are likely downstream factors regulated by bZIP60.

**Suppression of bZIP60 and SKP1 Reduces Local Infection and Systemic PVX Movement**

We inoculated plants with PVX-GFP and then monitored GFP fluorescence to determine if silencing bZIP60 interferes with the spread of virus infection throughout the plant. Table 1 shows that all plants that were pre-treated with buffer or TRV produced an average of 26-27 infection foci per leaf and became systemically infected with PVX-GFP by 5 dpi. bZIP60 silenced plants showed fewer infection foci (average of 18) and only 33% (4 of 12) became systemically infected with PVX-GFP at 5 dpi. We noted 75% of bZIP60 silenced plants became infected by 7dpi, and 100% were infected by 9 dpi (Table 1; Fig. 5A). Thus, silencing bZIP60 slowed the spread of virus infection to the upper leaves. This conclusion is further supported by immunoblot analysis performed to detect PVX coat protein (CP) in systemically infected leaves at 7dpi. PVX accumulation was greatly reduced in bZIP60-silenced plants in comparison to buffer or TRV pre-treated plants (Fig. 5B). These combined data indicate that bZIP60 is a contributing factor to optimum PVX accumulation in systemic tissues.

We also inoculated SKP1 silenced plants with PVX-GFP and then monitored GFP fluorescence to determine if SKP1 is vital for the spread of virus infection. There were fewer infection foci (average of 19) on SKP1-silenced plants than on buffer or TRV pretreated leaves (Table 1). This is comparable to the numbers of infection foci occurring on bZIP60-silenced plants. At 5 dpi, 50% (6 of 12) of SKP1-silenced plants were systemically infected with PVX-GFP (Table 1). By 7 dpi, all SKP1-silenced plants were systemically infected with PVX-GFP. Thus there is a slight delay in systemic infection compared to control plants (Table 1). Immunoblot analysis was performed to detect PVX coat protein (CP) at 7dpi, in systemically infected leaves and there was no change in comparison to control plants (Fig 5A).

With respect to SKP1 silenced plants, we observed higher GFP fluorescence in upper leaves although the immunoblot showed PVX accumulation in systemic tissues was unaltered. Given that SKP1 is a factor contributing to protein turnover, it is possible that silencing SKP1 reduced the turnover of GFP within infected cells. We have reported increased GFP
accumulation in GFP-expressing transgenic leaves treated with a cocktail of proteasome inhibitors, which points to the likelihood that GFP can be a target for the 20S and/or 26S proteasome (Mekuria et al., 2008). We also reported a 5-fold reduction in the steady state levels of GFP in PVX-GFP infected protoplasts that were treated with tunicamycin, indicating that GFP turnover may be regulated by UPR machinery (Ju et al., 2008). Tunicamycin is often used as a chemical stimulus of the unfolded protein response (Leborgne-Castel et al., 1999; Surjit et al., 2007). Thus it is reasonable to consider that the greater intensity of fluorescence may not be an indicator of higher virus titer.

To determine if bZIP60 and SKP1 contribute to virus accumulation in single cells, we delivered synthetic double strand RNAs (dsRNAs) targeting SKP1 or bZIP60 for silencing (Fig 5C). Protoplasts were harvested at 36 hpi and Northern analysis showed high levels of SKP1 and bZIP60 in untreated BY-2 protoplasts, but barely detectable levels of the same transcripts in protoplasts treated with dsRNAs. These data indicate that the dsRNAs can successfully knock down host gene expression. PVX-GFP transcripts were delivered to untreated and dsRNA treated protoplasts and northern analysis was carried out at 36 hpi. PVX genomic RNA accumulation was unaffected by silencing SKP1 but was significantly impeded in bZIP60 silenced protoplasts. These data indicate that bZIP60, but not SKP1, is a factor in virus replication.

Since we showed in Fig 4 that bZIP60 might regulate expression of SKP1, but knocking down each gene has a different outcome in isolated protoplasts it is arguable that SKP1 does not play the same roll in virus replication as bZIP60. bZIP60 may regulate other genes that directly affect PVX replication, in addition to contributing to the regulation of SKP1 expression. In plants, knocking down bZIP60 seem to contribute to reduce or delay virus systemic accumulation, although knocking down these genes have different affects on GFP intensity in systemic leaves (Fig 5, Table 1). While the data thus far argues that this gene network contributes to PVX infection, based on these limited experiments it is reasonable to propose that bZIP60 regulates more than one gene that contributes to optimum PVX infection. More studies are needed to elaborate the separate roles of bZIP60 and SKP1 in PVX infection.

**TGBp3 Expression Does Not Significantly Influence the Turnover of TGB2 or Replicase**
Many mammalian viruses regulate virus replication via UPR. They encode proteins that insert into the ER membrane and stimulate UPR which targets the viral replicase for degradation as a means to downregulate virus replication late in infection (Yu et al., 2006; Medigeshi et al., 2007). Since PVX replicase, TGBp2, and TGBp3 associate with the ER we considered the possibility that TGBp3 stimulates UPR as a means to regulate turnover of other PVX proteins in the ER. Also, given the differences in GFP intensity when expressed from the PVX genome in SKP1-silenced plants, it seemed reasonable to consider that TGBp3 might stimulate cellular UPR to regulate accumulation of other PVX proteins. To examine this hypothesis, TGBp3His and mycTGBp2 or TGBp3His and mycRep (replicase) were co-expressed in N. benthamiana leaves using agro-infiltration and immunoblot analysis was used to detect the epitope-tagged proteins at 3 d post infiltration (Fig. 6). Leaves were also infiltrated with A. tumefaciens as controls. Consistently high levels of TGBp3-His were detected when it was expressed alone or co-delivered with mycTGBp2 or mycRep. TGBp3 did not appear to have a significant impact on the accumulation of mycTGBp2 or mycRep.

Ectopic expression of TGBp3 Can Lead to Cell Death which can be alleviated by BiP overexpression

We re-examined leaves that were agro-infiltrated with plasmids expressing TGBp3 from the CaMV 35S promoter and noted microscopic necrotic lesions (Fig. 7A) which are absent from control agro-delivery of β-glucuronidase (GUS) coding sequences or A. tumefaciens alone. HR necrosis is evident from blue autofluorescence seen under UV light and ROS activity was detected using the fluorogenic probe 2′, 7′-dichloro-fluorescein diacetate (H2DCFDA) (Fig. 7A).

To determine whether BiP is directly responsible for TGBp3-related HR or represents a pathway branch that is induced by TGBp3 alongside the cell death signaling pathway, we over expressed the NbBLP-4 (BiP) coding sequence from the pBI121 plasmid. N. benthamiana leaves were infiltrated with A. tumefaciens containing pBI-NbBLP-4, pBI121 alone, or with buffer and immunoblot analysis was used to compare BiP protein levels among N. benthamiana leaves harvested 2 d after infiltration (Fig. 7B). The density of bands reporting BiP expression were 3-fold higher in NbBLP-4 infiltrated leaves than buffer treated leaves (data not shown). This level of overexpression is within the range reported at 3 dpi for PVX-infected leaves and agro-infiltrated leaves expressing TGBp3.
We agro-infiltrated leaves delivering BiP alone (pBI-NbBLP-4), TGBp3 alone, or a mixture of Agrobacterium expressing BiP and TGBp3 (Fig. 7C). BiP overexpression was sufficient to alleviate TGBp3 induced necrosis (Fig 7C). Using a UV lamp and H$_2$DCFDA staining, necrosis was seen only in TGBp3 expressing leaves. Co-delivery of BiP eliminated necrosis and evidence of ROS (Fig 7C). These data concur with earlier findings that BiP is upregulated upon pathogen invasion as a response to the increase in protein translation, but is not directly responsible for HR (Jelitto-Van Dooren et al., 1999). Importantly, the fact that necrosis is abrogated by BiP overexpression clearly demonstrates that TGBp3-related cell death is linked to ER stress. Given that Fig 4 shows bZIP60 is a factor regulating BiP expression as well as SKP1, these data argue that bZIP60 and BiP might play a role in regulating cytotoxic effects of PVX proteins during virus infection. Limiting protein cytotoxicity might be important for enabling optimal systemic virus spread by reducing tissue necrosis.

**DISCUSSION**

Here we report that bZIP60, several plant UPR related ER resident chaperones, and the Cullin co-chaperone SKP1 are induced following PVX infection or A. tumefaciens delivery of TGBp3 to N. benthamiana or Arabidopsis plants. Evidence that PVX infection, and ectopically expressed TGBp3 upregulates bZIP60, SKP1, and ER resident chaperones such as BiP is intriguing and provides the first clear evidence that a plant viral protein elicits UPR and a factor (SKP1) linked to proteasome-dependent pathways. Such comparisons of Arabidopsis and N. benthamiana gene expression in response to TGBp3 delivery are significant because they demonstrate that this is not a host-specific response and that there are general implications for host-virus interactions. Unfortunately, the incomplete representation of host genes on the microarrays and the lack of the complete N. benthamiana genome sequence hinder identification of orthologous genes or explain why certain genes such as bZIP factors, were not identified in Arabidopsis although they were identified in the potato microarrays. However, in a broader context, the data shows that members of gene families encoding ER resident proteins can be induced to similar levels (between 2 and 5.5 fold) in both species.

bZIP60 belongs to a class of membrane bound ER stress sensors that is responsible for up regulating genes involved in UPR (Supplemental Fig. S1) (Iwata and Koizumi, 2005; Urade, 2007; Lu and Christopher, 2008). bZIP60 is activated by ER stress and regulated by
intramembrane proteolysis. Cleavage of the full-length protein by a non-canonical proteolytic event releases the transcription factor from the ER (Iwata et al., 2008; Iwata et al., 2009). The truncated bZIP60 activates promoters containing cis elements, P-UPRE and ERSE, which are responsible for ER stress response, including activating its own transcription and BiP genes (Seo et al., 2008; Urade, 2009) (Fig. 1). bZIP60 regulated UPR is potentially a factor in promoting optimal PVX accumulation in infected protoplasts and plants by: a) reducing cytotoxicity that can lead to necrosis and; b) regulating expression of cellular factors contributing to virus infection. These conclusions are based on critical observations. First, our observations that agro-infiltration of \textit{NbBLP-4} eliminated TGBp3-induced HR confirmed a cytoprotective role for BiP in virus-infected leaves and for controlling TGBp3-induced ER stress (Fig. 1) (Iwata et al., 2008; Lu and Christopher, 2008; Urade, 2009). These experiments suggest that PVX employs the UPR machinery, via TGBp3 and BiP, to regulate cytotoxic damage to the cell as a means to promote virus spread. UPR is reported to be a component of important early responses to pathogen invasion in anticipation of the increase in protein synthesis along the ER, but is not directly responsible for defense gene induction (Jelitto-Van Dooren et al., 1999). In particular, BiP is a well known component of cellular cytoprotective responses to alterations in the ER or accumulation of misfolded proteins and controls the status of certain UPR transmembrane signal transducers (\textit{e.g.} IRE1, PERK, and ATF6) (Tardif et al., 2004; Zhang and Kaufman, 2006). Lebourne-Castel et al. (1999) were the first to demonstrate that mild overexpression of BiP (\textit{NbBLP-4}) in transgenic plants restores ER homeostasis and protects plants from ER stress (Leborgne-Castel et al., 1999; Costa et al., 2008). Importantly, \textit{AtHSP70} induction is also linked to plant protein overexpression. BiP is a member of the HSP70 multigene family, and a subset of cytoplasmic \textit{AtHSP70} genes (\textit{HSC70-1}, -2, -3, and \textit{HSP70} but not \textit{AtHSP70B}) are induced as part of a general response to viral protein accumulation (Whitham et al., 2003; Aparicio et al., 2005). Thus HSP70 may contribute to modulating cellular stresses during virus infection in a manner that is reminiscent of the UPR (Aparicio et al., 2005). Our study contrasts with the prior work on HSP70 by presenting several experimental outcomes which point to TGBp3 as a specific inducer of BiP expression.

To support the notion that UPR is a factor regulating disease, we inoculated \textit{bZIP60} and \textit{SKP1}-silenced plants and protoplasts with PVX-GFP. We reported fewer infection foci on \textit{bZIP60} and \textit{SKP1}-silenced \textit{N. benthamiana} plants, suggesting that PVX infection was attenuated
by the greatly reduced bZIP60 or SKP1 expression. The reduced number of green fluorescent infection foci (Table 1) and reduced PVX genomic RNA accumulation in protoplasts correlated with the dramatic reduction in bZIP60 transcript accumulation. These data suggest that bZIP60 is a factor contributing to PVX-GFP replication in protoplasts and N. benthamiana leaves. The partial inhibition of bZIP60 expression in N. benthamiana plants did not compare to the inhibition seen in protoplasts. Therefore new research tools, are needed improve knockdown of bZIP60 in N. benthamiana plants or other hosts of PVX to examine the contribution of bZIP60 to promoting long distance PVX spread. It is worth considering bZIP60 as a target for developing a transgenic approach to virus resistance.

SKP1 is an essential component of the SCF family of E3 ubiquitin ligases which provides substrate ubiquitination preceding proteasome-mediated degradation (Cardozo and Pagano, 2004; Petroski and Deshaies, 2005). Typical substrates are cellular proteins crucial for eukaryotic physiology and defense. In Fig. 2, we report that PVX infection and TGBp3 upregulates SKP1 mRNA expression, but it is not clear if increased SKP1 expression is necessary to degrade viral proteins or simply to alleviate congestion of proteins in the ER by enhancing protein turnover. We also noticed that SKP1 is upregulated by TGBp2 at 5 dpi although TGBp2 does not appear to be impacted bZIP60 expression. We also showed that silencing bZIP60 can alter expression of SKP1 in the absence of a viral inducer which suggests that these genes are linked in a pathway. But failure to completely shut down SKP1 expression, combined with evidence that TGBp2 can induce SKP1 raises the possibility that SKP1 may be controlled by additional factors and may not be completely controlled by bZIP60. Further analyses are needed to detail the relationship of these genes. This would require cloning the SKP1 promoter and analyzing the elements that control gene expression. For example, it would be interesting to learn if SKP1 promoter has P-UPRE and ERSE elements and may be recognized by other bZIP transcription factors.

Evidence linking TGBp3 to SKP1 is exciting given recent reports linking proteasomal activities to systemic virus movement and to the function of certain of plant viral silencing suppressor proteins. For example, RPN9 is a proteasomal subunit whose expression is required for systemic movement of Tobacco mosaic virus and Turnip mosaic virus. Silencing RPN9 seemed to generally impede virus systemic movement, although the protein also seems to play a role in appropriate vascular development (Jin et al., 2006). More closely related to this work is
evidence that the Beet western yellows virus (BWYV) P0 silencing suppressor has an F-box domain and directly binds SKP1 orthologs in Arabidopsis, although the cellular components of the silencing machinery that are targeted by the SCF E3 ubiquitin ligase are not known (Baumberger et al., 2007). However, silencing SKP1 in N. benthamiana caused plants to become resistant to BWYV infection indicating the relationship of P0 and SKP1 is vital for virus spread. We report here that silencing SKP1 reduced the number of infection sites on inoculated leaves and the number of infected plants (Table 1) suggesting that it plays a role in PVX infection. Moreover, the PVX TGBp1 silencing suppressor targets AGO1, which is the effector nuclease of RNA silencing machinery, for degradation via the proteasome (Chiu et al., 2010). Thus combining this work with previous reports of TGBp1, it is possible that TGBp3 upregulates UPR and components of the ubiquitin-proteasome pathway to enable degradation of AGO1 mediated by TGBp1. Thus the TGBp1 and TGBp3 proteins might act in concert to regulate host defense and stress responses in a manner that renders plants more susceptible to PVX infection. Further research is needed to determine the link between TGBp1, TGBp3 and proteasomal degradation of cellular components of the silencing machinery.

Moreover, this outcome suggests a role for the UPR in promoting virus spread and raises the question of how do bZIP60, UPR, as well as cellular events required to maintain ER homeostasis, regulate systemic PVX accumulation? There are three possible explanations. First, bZIP60 could play a direct role in PVX infection that is unrelated to its role in UPR induction. This explanation seems unlikely given that bZIP60 is responsible for up regulation of ER resident chaperones such as BiP (Iwata and Koizumi, 2005; Lu and Christopher, 2008), and we show that BiP plays a role in suppressing TGBp3-related ER stress. Second, bZIP60 might upregulate another gene whose protein product is a positive factor in promoting virus replication and movement. Further experiments are needed to identify additional bZIP60-regulated factors and assess their role in PVX movement. Third, bZIP60 might be required to enhance cellular protein folding abilities (perhaps by increasing BiP expression), proteasomal function for degradation of AGO1, and ER membrane synthesis necessary for optimal virus accumulation. This latter possibility is based on the flavivirus model. The nonstructural proteins of JEV and DEN-2 trigger the ER resident sensors which lead to signaling pathways that enhance cellular protein folding abilities, ER membrane synthesis, and up regulation of the secretory system (Urano et al., 2000). These events are necessary to manage the increase in protein translation.
resulting from virus infection and provides further membranes needed for replication and maturation (Yu et al., 2006). During PVX infection, expansion of the ER network is known to be important for virus infection. Cells treated with cerulenin, an inhibitor of membrane synthesis, supported reduced virus replication (Bamunusinghe et al., 2009). Thus the preliminary data point to the possibility that PVX, similar to flaviviruses, triggers UPR to enhanced cellular protein folding abilities and ER membrane synthesis. If SKP1 expression is regulated by bZIP60 then there is additional regulation of the proteasomal pathway that might be important for effective silencing suppression mediated by TGBp1. These combined events could be necessary to promote virus cell-to-cell movement.

Similarly, build-up of viral proteins in the ER or the retention of inefficiently folded viral envelope proteins in the ER is cytotoxic and leads to UPR initiation by mammalian viruses such as HCV, JEV, human cytomegalovirus, and bornavirus (Chan and Egan, 2005; Williams and Lipkin, 2006). In these examples, viral proteins trigger ER stress in a manner that leads to cell death only when the protein load in the ER exceeds the folding capacity induced by ER stress. When we compare the effects of expressing TGBp3 at various levels, it becomes worth considering that the amounts of TGBp3 expressed during PVX infection are tightly regulated to avoid damaging the cells. Given that TGBp3 is expressed from the PVX genome via a subgenomic RNA at low levels, this may be necessary to promote virus spread by preventing cytotoxic cell death.

There are intriguing similarities between PVX TGBp3 and the HIV Vpu protein. Both proteins are expressed from bicistronic mRNAs, and they have low molecular weights with single transmembrane domains that insert into the ER. Vpu binds to the cellular CD4 protein in the ER and recruits the human F-box protein βTrCP targeting CD4 for degradation via the ubiquitin-proteasome pathway. CD4 is a cell surface receptor required for HIV uptake into cells and the process of dislocation and degradation of CD4 in the ER reduces the number of available receptors at the cell surface and is important to free HIV gp160 in the ER for virus maturation and trafficking (Bour et al., 1995; Schubert et al., 1998; Malim and Emerman, 2008; Nomaguchi et al., 2008). It is worth considering that TGBp3 might function to bind cellular proteins and recruit them to the ubiquitin-proteasome pathway. PVX TGBp3 might function in the ER to down regulate host factors contributing to virus replication or early stages of infection during their translation which could be essential for maintaining virus infection and promoting cell-to-
cell spread. Further experiments are needed to identify host proteins interacting with TGBp3 and its ability to associate with components of the SCF complex.

CONCLUSION
For the last 15 years plant virologists have reported viral movement proteins embedded in the ER. Until now researchers have viewed the ER as a location for assembly and lateral transport of movement complexes toward plasmodesmata, but there have been no reports indicating a role for the ER or UPR in promoting plant virus spread. This is in contrast to significant advances on this topic that have been made in mammalian virus research. The data presented in this study point to a new role for the ER in regulating plant virus movement. We provide the first evidence linking UPR to systemic accumulation of PVX and raise the possibility that bZIP60 is an important factor in PVX infection. This study of the ER-resident PVX TGBp3 protein will open the door to further examination of whether PVX employs machinery similar to the ER-resident proteins encoded by flaviviruses or retroviruses, such as HIV, to modulate various ER stress responses as a means to cope with robust viral protein synthesis (Tardif et al., 2004; Chan and Egan, 2005; Medigesi et al., 2007; Alwine, 2008; Surjit and Lal, 2008), increase membrane biosynthesis needed for virus replication and maturation, prevent superinfection, and modulate cell death functions.

Materials and Methods
Plasmids and bacteria strains
pGR208 is a binary vector containing PVX-GFP genome was obtained from Dr. P. Moffett (University of Sherbrooke). pGR208 is deliverable to plants by agro-infiltration.

A 6x-His tag (underlined) was introduced at the 3’ end of the PVX TGBp3 coding sequence in pTXS-GFP plasmids using the Quick-change II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), forward primers (5’-GTTGACGGTTAAGTTTCACCATCACCATCACATGATACTCGAAAG-3’) and reverse primers (5’-CTTTTCAGATATGATGGATGGACTACATTGACTCGAAAG-3’). The reaction products were transformed into E.coli XL10-Gold.

A.tumefaciens deliverable binary vectors were prepared using pMDC32 plasmids and Gateway Technology with Clonase II (Invitrogen, Carlsbad, CA). The pMDC32 contains the
CaMV 35S promoter. To generate pMD32-TGB3His, the TGB3His DNA fragment was first amplified using with attB1 primers (GGGGACAAAGTTTGTACAAAAAACACCGCTTCGG ATCCATGGAAGTAATACATATC) and attB2 primers containing 6x-His tag (underlined), (5-GGGGACCACTTTTGATACAAAAAGCTGGGTCTCAGTGTTGATGGATGATG GAAACTTAAACCGTGTTCAAC). To generate pMD32-TGBp1 and CP, PCR fragments were amplified using attB1 primers (GGGGACAAAGTTTGTACAAAAAACGAGCTGTATG GATATTCTCATCAGTAG; GGGGACAGTTTTGTACAAAAAACGAGCTGTATGTC AGCACCAGCTAGC) and attB2 primers (GGGGACCACTTTGATACAAAAAACGAGCTGGGTGCT ATGGCCC TGCGCGGACAT; GGGGACCACTTTGATACAAAAAACGAGCTGGGTGTTA TGGTGGTGTTG AGAGTGAC). The DNA fragments were incubated with pDONR/zeo and BP clonase II for one h. Then pMDC32 binary vector (obtained from Dr. R. Sunkar, Oklahoma State University) and LR clonase II were added to the reaction mix and incubated for one h. The reaction products were transformed to One Shot OmniMax2-T1 competent E.coli cells. After sequencing confirmation of the derived plasmids, the pMDC32-TGBp3his plasmid was used to transform to A. tumefaciens strain GV2260. A set of plasmids were also prepared replacing the CaMV35S promoter with the NOS promoter. 307 nucleotides of NOS promoter is amplified from pBI121 plasmid using the forward primer: GCAAGCTT GATCATGAGCGGAGAAT TAAG (Hind III site is underlined) and a reverse primer: GCGGTACC AGATCCGGTGCA GATTATTTGG (Kpn I site is underlined). The PCR fragment was cloned into pMDC-p3H between Hind III and Kpn I to replace its CaMV 35S promoter.

The A. tumefaciens deliverable pGWB21 binary vector (obtained from Dr. T. Nakagawa, Shimane University, Japan) which includes a 11x myc tag at the 5’ end of inserted open reading frames. PVX TGBp2 and replicase coding sequences were cloned into pGWB21 using the same Gateway Technology described above. To generate pGWB21-mycTGB2, TGB2 was PCR amplified using forward attB1 primers containing the myc tag (underlined) (5’-GGGGACAAAGTTTGTACAAAAAACGAGCTGGGTCTCAGTGTTGATGGATGATG GAAACTTAAACCGTGTTCAAC). To generate pGWB21-mycRep, the PVX replicase was PCR amplified using forward attB1 primers containing the myc (underlined) tag (5’-GGGGACAAAGTTTGTACAAAAAACAGCAGCTTCGGATCC ATGGTACAAAAAACAGGCTTTCCGATCC ATGGAAACAAAAATTTTCTAAGA TCTG TCCGCGCAGGGCCCATAGG-3’) and reverse attB2 primers (5’-GGGGACCACTTTTGATACAAAAAGCTGGGTCTCAGTGTTGATGGATGATG GAAACTTAAACCGTGTTCAAC). To generate pGWB21-mycRep, the PVX replicase was PCR amplified using forward attB1 primers containing the myc (underlined) tag (5’-GGGGACAAAGTTTGTACAAAAAACAGCAGCTTCGGATCC ATGGTACAAAAAACAGGCTTTCCGATCC ATGGAAACAAAAATTTTCTAAGA TCTG TCCGCGCAGGGCCCATAGG-3’).
TGAACAGAAACTTATTTCTGGAAGAAGATCTG
GCCAAGGTGCGCGAGGTT-3') and reverse attB2 primers (5’-GGGGACCACCTTTGTACAAGAAA
GCTGGGTCTTAAAG AAA
GTTTCTGAGGCG-3’). After sequencing confirmation, pGWB21-mycTGBp2 and pGWB21-
mycRep were transformed to A. tumefaciens strain GV2260.

A. tumefaciens LBA4404 containing pBI- BLP4 was prepared by inserting the coding
sequence between XbaI and SacI restriction sites of pBI121 plasmids (Jefferson et al., 1987).
Total RNA extracted from N. benthamiana leaves using Trizol Reagent (Invitrogen, Carlsbad,
CA) and treated with DNase I (Promega, Madison, WI). NbBLP-4 coding sequence (Accession
No FJ463755) was synthesized with Superscript Reverse Transcriptase III (Invitrogen) and
amplified by Pfu Turbo DNA polymerase (Stratagene). NbBLP-4 cloning primers are designed
based on NtBLP-4 (Accession No. X60057) (Table S1). The NbBLP-4 showed greater than 98%
homology with the NtBLP-4 cDNA (Leborgne-Castel et al., 1999). NbBLP4 cDNA was cloned
into pGEM-T Easy vector (Promega) and sequenced with M13 primers. The plasmid
pRTL2.TGBp3-GFP was prepared previously (Samuels et al., 2007). TGBp3Dm1-GFP was PCR
amplified using primers containing NcoI and BamHI restriction sites and then inserted into
pRTL2 plasmids.

A. tumefaciens strain GV2260 containing TRV1 plus TRV2-NbSKP1, TRV2-bZIP60,
TRV2-PDS or TRV2-GFP was kindly from Dr. S. Dinesh-Kumar (Yale University) (Liu et al.,
2004; Bhattarai et al., 2007). NtbZIP60 gene fragment (168-776nt of NtbZIP60 coding
sequence) was cloned into pDONR/zeo and pTRV2 gateway vectors with Gateway Technology
Clonase II (Invitrogen). forward primer attB1 (5’-GGGGACAAGTTTGTACAAAAAAGCAGG
CTGTCGGTACGT GGCTGTC-3’ and reverse primer attB2 (5’-GGGGACCACCTTTTG
CTGTCGGTACGT GGCTGTC-3’).

Plant Materials and Inoculations

N. benthamiana and Arabidopsis plants were used. Purified virus was prepared from
infected N. benthamiana plants using the traditional methods, and suspended in 0.01M
phosphate buffer (pH7.0)(Shadwick and Doran, 2007). Virus concentration (C) was determined
by measuring OD_{260}, and calculated by the formula C=OD_{260}/3.0. Aliquots of viruses are stored
in -80°C and then 30 µg/ml virus was used for each inoculation.
Agro-infiltration for plasmid or TRV delivery to *N. benthamiana* or *Arabidopsis* leaves was performed with 1 ml needle-free syringe according to published protocols (Liu et al., 2002). *A. tumefaciens* LBA4404 or GV2260 infiltrations were conducted using ten plants for each treatment and infiltration medium (buffer) was used as negative control. *A. tumefaciens* cultures were collected by centrifugation, and resuspended in agro-infiltration solution (10mM MgCl₂, 10mM MES, pH 7.0, 200µM acetosyringone). The suspension was adjusted to OD₆₀₀ =1.0, 0.1, or 0.01 and infiltrated to *N. benthamiana* leaves with 1ml needle-free syringe. *N. benthamiana* plants were grown to a four-leaf stage for infiltration. GV2260 cells were also infiltrated to leaves serving as the negative control.

**H₂DCFDA Staining of Leaf Segments**

*N. benthamiana* were agro infiltrated and ROS activity was detected using the fluorogenic probe H₂DCFDA (Mahalingam et al., 2006). For visual assessment of ROS activity, leaf samples were treated with 50 µM H₂DCFDA for 20 min and observed using a Nikon E600 epifluorescence microscope.

**Immunoblot Analyses**

For immunoblot analysis of virus infected or agro-infiltrated leaves, 0.3g treated leaf samples were harvested from inoculated leaves at 5 dpi or upper leaves at 8-10 dpi. Total protein was extracted from leaves by grinding samples with extraction buffer (4M urea, 4% SDS, 0.2M DTT, 20% glycerol, 0.2M Tris-HCl, pH6.8, and 0.04% bromophenol blue) (Draghici and Varrelmann, 2009) or standard protein extraction buffer (100mM Tris-HCl, pH7.5, 10mM KCl, 0.4M sucrose, 10% glycerol, 10µM PMSF) (Sambrook, 1989) and quantified using Bradford Reagent (Sigma-Aldrich, St. Louis, MO). Thirty µg, protein for each sample was load onto 4–20% precast gradient or 10% SDS-PAGE (Bio-Rad), electroblotted to Hybond-P (GE Healthcare, Piscataway, NJ). Blots were probed with BiP (GRP78) antiserum (Affinity BioReagents, Golden, CO), 5x-His monoclonal IgG (Qiagen, Valencia, CA), c-Myc monoclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA), PVX coat protein (Agdia, Elkhart, IN) or GFP polyclonal antiserum (Affinity BioReagents). HRP conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) served as the secondary antiserum using ECL Advanced Western Blotting Kit (GE Healthcare). Blots were exposed to film for 10-60 seconds. Film was scanned using
Alpha Image imaging system (Alpha Innotech. San Leandro, CA) and the reverse image was recorded. Densitometric analysis was performed by Alpha Ease FC software (Alpha Innotech). Films are scanned and images are cropped with scanner CanonScan 9950F and associated program Arcsoft Photo Studio 5 (Canon USA).

**qRT-PCR Analysis of Infected Leaves and Semi-quantitative RT-PCR of Silenced Plants**

Mock inoculated (treated with agro-infiltration buffer) was used as a control and calibrator sample. SV Total RNA Isolation kit (Promega Corp.) was used to extract total RNA from samples. The first strand cDNA was synthesized by Superscript reverse transcriptase III (Invitrogen) using hexamer random primers. qPCR was carried out using 25 μl reactions and 100 to 900 nM primers designed using the coding sequences for known Arabidopsis, N. tabacum or N. benthamiana genes (Supplemental Table S1). Initial RT-PCR tests of the gene specific primers confirmed their ability to amplify single bands of the predicted sizes from N. benthamiana cDNA. 25ng cDNA was used to perform qPCR using the Power SYBR Green II Master Mix and ABI 7500 PCR machine (Applied Biosystem, Foster City, CA). Reactions were incubated first at 95 °C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Efficiencies of all primers were verified by normal RT-PCR and gel electrophoresis. The comparative C_T method was employed for relative quantitation of gene expression following virus treatments. qRT-PCR efficiencies were determined by control amplifications using 0.01, 0.1, 1, 10, 100 ng of template cDNA. Duplicate PCR reactions for each sample were carried out and averaged. The comparative C_T method employs the formula 2 ^-ddCT where the values of the endogenous control (18S RNA) and calibrator (constant quantity of healthy sample template) and are subtracted from the target sample value to provide the ddC_T value. The 2 ^-ddCT represents the fold of RNA accumulation.

Semi-quantitative RT-PCR NbSKP1 and bZIP60 primers were designed to anneal outside the target sequence of virus-induced gene silencing (Liu et al., 2002). To detect SKP1 or bZIP60 silencing effect, total RNA was extracted from agro-infiltrated N. benthamiana leaves at 14 days post-infiltration with SV Total RNA Isolation System (Promega). The first strand cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen), 1μg total RNA and 100μM 6-mer random primers. bZIP60 semi-quantitative RT-PCR was performed with bZIP60 primers forward 5'-CCTGCTTTTGTTTCATGGGCATCAT-3' (672-695 nt), reverse 5'-
CACATCACA ATT CCCAAATAATG-3’ (877-900 nt). For amplification of NbSKP1 we employed forward primers (5’-TGACATGCCAGACAGTTGCAGACA-3’) (303-326 nt), and reverse primers (5’-TTGCATCTCATCTCGGTCT-3’) (439-462 nt). *N. benthamiana* actin primers forward (5’-AAAGACCAGCTCATCCGTGGAGAA-3’), and reverse primers (5’-TGTGGTTTCATGAATGCCAGC-3’) were used to amplify actin as the internal control. Semi-quantitative RT-PCR was performed with same protocols described above.

**Preparation of BY-2 protoplasts, dsRNA delivery, and northern analysis.**

BY-2 protoplasts are prepared and transfected as previously described (Lee et al., 2008). Two µg of *NbSKP1* or *NtbZIP60* dsRNAs, and 25 µg PVX-GFP transcripts were delivered to 1 x 10^6 protoplasts. Transfected BY-2 cells were incubated at 25ºC and then total RNA was extracted at 48 h using TRIzol Reagent (Invitrogen). 15 µg total RNA was subject to Northern detection with the North-2-South Chemiluminescent Hybridization and Detection kit (Pierce Biotechnology, Rockford, IL).

PVX-GFP transcripts were prepared using standardized protocols that were previously reported (Bamunusinghe et al., 2009). dsRNAs were prepared as previously described (Silva et al., 2010) (Qi et al., 2004). A PCR fragment of *NbSKP1* coding sequence (21-457 nt) and a fragment of *NtbZIP60* coding sequence (168-776 nt) were cloned into pGEM-T Easy (Promega). The plasmids were linearized using *Spe I* and positive sense transcripts were synthesized using RiboMAX Large Scale RNA Production System-T7 (Promega). Plasmids were also linearized using *Nco I* and then negative sense transcripts were synthesized using RiboMAX Large Scale RNA Production System-SP6 (Promega). DNA was removed by digestion for 15 min using DNAse I. Transcripts were precipitated and then resuspended in RNAse-free ddH2O. Equal amounts of positive and negative strand RNAs were mixed in annealing buffer (100 mM potassium acetate, 4 mM MgCl2 and 60 mM HEPES–KOH, pH 7.4), and incubated overnight at 37ºC to produce dsSKP1 or dsbZIP60 RNAs.

Probes for northern blots were prepared by adding 100 ng of NbSKP1, NtbZIP60 or PVX CP PCR fragments to a random priming reaction (North-2-South Biotin Random Prime Labeling kit, Pierce Biotechnology). Blots were incubated overnight with each probe at 55ºC and developed using Kodak Biolight film.
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Figure Legends

Figure 1. Boxplots representing qRT-PCR analysis transcript levels in PVX infected N. benthamiana leaves. Genes analyzed are indicated above each graph. Box plots represent the range of values obtained for 20 samples and the variability of gene expression. The boundaries of each box represent the lower 25th and upper 75th percentiles, and the horizontal line within the box represents the median values (i.e. 50th percentile). The spacing of components within the box indicates the degree of dispersal, or skewness, in the data. The lines at the top and bottom of the box (whiskers) represent the sample minimum and maximum. Longer lines at the top indicate a positive skewness. Outliers are indicated by “x”. Kruskal-Wallis test of the equality of medians reported for bZIP60 p < 0.0001, BiP p < 0.001, PDI p = 0.007, CAM p = 0.0143, CRT p = 0.007, SKP1 p <0.0001.

Figure 2. TGBp3 induction of BiP, CAM, CRT, PDI, SKP1, and bZIP60 transcripts following agro-infiltration. (A) Diagrammatic representation of constructs used in this study. Black arrows indicate CaMV 35S promoter and light gray arrow indicates NOS promoter. Boxes represent open reading frames. Name for each construct is listed on the right. Brown bars indicate myc or His tags. (B) Immunoblots containing protein extracts from N. benthamiana leaves that were infiltrated with buffer (0; lane 1); or A. tumefaciens containing PVX-GFP (P); mycRep (mycR); mycTGBp2 (myc-p2); TGBp3His (p3His); TGBp1 (p1); CP. The final immunoblot at the bottom shows PVX –GFP and PVX-p3His. The latter contains His tag fused
to TGBp3 and immunoblot was carried out using CP antisera. Immunoblot shows CP levels are comparable in systemic tissues at 7 dpi indicating that the His tag is not deleterious to virus accumulation. The antisera used for protein detection are identified below the blots. The PVX used in these experiments has the His-tag fused to TGBp3 and therefore His antisera can detect TGBp3 in the PVX genome. (C) Leaves were agro-infiltrated, total RNA was extracted at 2 or 5 dpi, and qRT-PCR was carried out. Bars represent the average of three replicate samples.

Figure 3. Induction following delivery of TGBp3 to Arabidopsis and using alternative ectopic promoters. Top of each panel shows immunoblot probed with His-antisera. Immunoblot in (A) contains protein extracts from Arabidopsis leaves that were infiltrated with buffer (M), A. tumefaciens only (0), or A. tumefaciens containing plasmids expressing TGBp3-His. Immunoblot in (B) contains protein extracts from N. benthamiana leaves which were infiltrated with A. tumefaciens containing TGBp3 fused to fused to either the NOS (lanes 2-5) or CaMV 35S promoter (lanes 6-9) promoter. These immunoblot verify protein expression in planta. Coomassie stained gel located below the immunoblot shows equal sample loading on the gel. (A) Arabidopsis leaves were infiltrated with buffer (mock), A. tumefaciens only (Agro), or A. tumefaciens expressing 35S-TGBp3. Total RNA was extracted at 2 or 5 dpi and qRT-PCR was carried out. The average of three replicate samples is represented by each bar. ANOVA was used to verify TGBp3 induced higher levels of host transcripts than other treatments at that time point (p<0.05). (B) N. benthamiana leaves were infiltrated with buffer (mock) or dilutions of A. tumefaciens containing TGBp3 fused to either the CaMV35S or Nos promoters.

Figure 4. Effects of TRV-bZIP60 on expression of bZIP60, BiP, and SKP1. Semi-quantitative RT-PCR conducted to verify silencing following TRV-VIGS treatment. The name of the gene analyzed by RT-PCR is listed at the top left of the panel. The sizes in base pairs (bp) of the DNA ladder (L) are indicated on the left. The bottom of each lane indicates the number of PCR cycles performed. PCR bands representing bZIP60, SKP1, and actin (internal control) after 25 cycles are shown for healthy and TRV-treated samples. Below actin are gel panels showing the outcomes of semi-quantitative RT-PCR detecting BiP or SKP1 on bZIP60 silenced plants. Bands representing BiP are not seen until 45 cycles and SKP1 bands appear at approximately 35 cycles.
Figure 5. TRV-VIGS silenced *N. benthamiana* plants were inoculated with PVX-GFP. (A) Images of systemic PVX-GFP infection at 7 dpi using a hand held UV lamp. Some plants were pre-treated with TRV, TRV-bZIP60, and TRV-SKP1 treated plants and then with PVX-GFP at 14 d following TRV delivery. Lower panel insert shows bZIP60-silenced plants with PVX-GFP fluorescence in systemic leaves at 9 dpi. (B) Immunoblot analysis confirms PVX coat protein (CP) in infected plants at 7 dpi. Treatment with buffer (0), TRV empty vector, TRV-bZIP60, or TRV- SKP1 is indicated above each pair of lanes. Coomassie blue stained gel below the immunoblot shows equal sample loading. (C). Northern analysis of BY-2 protoplasts at 36 hpi following transfection with PVX-GFP (P) transcripts and double strand (ds) RNAs used to knock down *NbSKP1* or bZIP60 expression. Top of each lane indicates BY-2 protoplasts that are untreated (0), treated with PVX or PVX plus dsRNAs, or dsRNAs alone. Labels on the right indicate RNA probe. Ethidium bromide stained gel image of rRNA is included below each northern. dsRNAs successfully knocked down SKP1 and bZIP60 expression in BY-2 protoplasts. PVX-GFP accumulation is limited in bZIP60-silenced protoplasts but not SKP1-silenced protoplasts.

Figure 6. Immunoblot analysis following agro-infiltration with combinations of plasmids expressing TGBp3His, mycTGBp2, mycRep. (A) *N. benthamiana* leaves infiltrated with a suspension of *A. tumefaciens* containing an empty vector (Agro), TGBp3His, mycTGBp2, or mycTGBp2 plus TGBp3His. Top panels shows immunoblot probed with myc antiserum. The second panel shows an immunoblot probed with His antiserum. Coomassie stained gel verifies equal loading of samples. (B) *N. benthamiana* leaves infiltrated with a suspension of *A. tumefaciens* containing an empty vector, TGBp3His, mycRep, or mycRep plus TGBp3His. The immunoblot in the top panel was probed with myc antiserum and the bottom panel was probed with His antiserum. Coomassie stained gel shows equal loading of samples.

Figure 7. Overexpression of BiP alleviates TGBp3-induced cell death. (A) *N. benthamiana* leaves infiltrated with *A. tumefaciens* alone, containing a GUS construct, or TGBp3 fused to CaMV 35S promoter. Bright field image shows necrotic flecks. Scale bar equals 50 μm. (B)
Immunoblot detection of BiP in leaf extracts at 2 d post infiltration. The image shows bright bands corresponding to immunodetected proteins. Films were scanned and reverse images were recorded for high resolution visualization of bands. Coomassie Blue stained membrane shows equal amounts of protein loaded in each lane. (C) N. benthamiana leaves infiltrated with A. tumefaciens listed on the left of each pair of panels. First column shows necrosis seen under UV lamp. Second column of panels show similar tissues following treatment with H$_2$DCFDA stain which detects ROS activity.

Table 1. Local and systemic PVX infection on silenced plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Avg. # foci/leaf$^a$</th>
<th># Infected at 5 dpi$^b$</th>
<th># Infected at 7 dpi$^b$</th>
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<tbody>
<tr>
<td>Buffer+ PVX-GFP</td>
<td>26±9</td>
<td>8/8</td>
<td>8/8</td>
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<tr>
<td>TRV+ PVX-GFP</td>
<td>27±11</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td>TRV-bZIP60+ PVX-GFP</td>
<td>18±10</td>
<td>4/12</td>
<td>9/12$^c$</td>
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<tr>
<td>TRV-SKP1+ PVX-GFP</td>
<td>19±8</td>
<td>6/12</td>
<td>12/12</td>
</tr>
</tbody>
</table>

$^a$ The average numbers of green fluorescent infection foci were determined using 16-19 leaves at 5 dpi.

$^b$ Total numbers of systemically infected plants relative to the total number of plants that were inoculated. Plants were scored based on the presence of systemic disease and green fluorescence in the upper leaves.

$^c$ All bZIP60 silenced plants were systemic at 9 dpi.
Figure 1. Boxplots representing qRT-PCR analysis transcript levels in PVX infected *N. benthamiana* leaves. Genes analyzed are indicated above each graph. Box plots represent the range of values obtained for 20 samples and the variability of gene expression. The boundaries of each box represent the lower 25th and upper 75th percentiles, and the horizontal line within the box represents the median values (i.e. 50 percentile). The spacing of components within the box indicates the degree of dispersal, or skewness, in the data. The lines at the top and bottom of the box (whiskers) represent the sample minimum and maximum. Longer lines at the top indicate a positive skewness. Outliers are indicated by “x”. Kruskal-Wallis of the equality of medians reported for bZIP60 p < 0.0001, BiP p < 0.001, PDI p = 0.007, CAM p = 0.0143, CRT p = 0.007, SKP1 p < 0.0001.
Figure 2. TGBp3 induction of BiP, CAM, CRT, PDI, SKP1, and bZIP60 transcripts following agro-infiltration. (A) Diagrammatic representation of constructs used in this study. Black arrows indicate CaMV 35S promoter and light gray arrow indicates NOS promoter. Boxes represent open reading frames. Name for each construct is listed on the right. Brown bars indicate myc or His tags. (B) Immunoblots containing protein extracts from N. benthamiana leaves that were infiltrated with buffer (0; lane 1); or A. tumefaciens containing PVX-GFP (P); mycRep (mycR); mycTGBp2 (myc-p2); TGBp3His (p3His); TGBp1 (p1); CP. The final immunoblot at the bottom shows PVX-GFP and PVX-p3His. The latter contains His tag fused to TGBp3 and immublot was carried out using CP antisera. Immunoblot shows CP levels are comparable in systemic tissues at 7 dpi indicating that the His tag is not deleterious to virus accumulation. The antisera used for protein detection are identified below the blots. The PVX used in these experiments has the His-tag fused to TGBp3 and therefore His antisera can detect TGBp3 in the PVX genome. (C) qRT-PCR was carried out 2 dpi and 5 dpi, and qRT-PCR was carried out. Bars represent the average of three replicate samples.
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