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SHORT COMMUNICATION

Binding immunoglobulin 2 functions as a proviral factor for potyvirus infections in *Nicotiana benthamiana*

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Abstract

Infection of viruses from the genera *Bromovirus*, *Potyvirus*, and *Potexvirus* in *Nicotiana benthamiana* induces significant up-regulation of the genes that encode the HSP70 family, including binding immunoglobulin protein 2 (BiP2). Three up-regulated genes were knocked down and infection assays with these knockdown lines demonstrated the importance of the *BiP2* gene for potyvirus infection but not for infection by the other tested viruses. Distinct symptoms of cucumber mosaic virus (CMV) and potato virus X (PVX) were observed in the *BiP2* knockdown line at 10 days postagroinfiltration. Interestingly, following inoculation with either soybean mosaic virus (SMV) or pepper mottle virus (PepMoV) co-expressing green fluorescent protein (GFP), neither crinkle symptoms nor GFP signals were observed in the *BiP2* knockdown line. Subsequent reverse transcription-quantitative PCR analysis demonstrated that knockdown of *BiP2* resulted in a significant decrease of SMV and PepMoV RNA accumulation but not PVX or CMV RNA accumulation. Further yeast two-hybrid and co-immunoprecipitation analyses validated the interaction between BiP2 and nuclear inclusion protein b (NIb) of SMV. Together, our findings suggest the crucial role of BiP2 as a proviral host factor necessary for potyvirus infection. The interaction between BiP2 and NIb may be the critical factor determining susceptibility in *N. benthamiana*, but further studies are needed to elucidate the underlying mechanism.

KEYWORDS
BiP2, host factor, infection, NIb, *Nicotiana benthamiana*, potyvirus

Managing disease incidences caused by plant viruses is crucial for securing global crop production. The generation and cultivation of resistant cultivars continue to be the most effective ways to control outbreaks and the spread of plant viruses in crop-growing areas. This approach, however, may not be practical for the long-term management of multiple virus diseases given the rapid mutations of the plant viruses that lead to the emergence of new resistance-breaking strains (Ahangaran et al., 2013; Choi et al., 2005; Chowda-Reddy et al., 2011; Gagarinova et al., 2008). The development of various techniques in plant virology within the past few decades may provide an alternative approach for more durable and effective management of plant virus diseases. One of the most promising strategies is manipulating the host factors required for plant virus infection (Hashimoto et al., 2016). A single host factor that affects multiple plant virus infections is favourable in this approach.

Kristin Widyasari and John Bwalya contributed equally to this work.

Plant viruses are obligate parasitic microbes with a relatively small genome that encodes only a limited number of proteins and thus they depend on host plant machinery to complete their infection cycles. The interaction between viral proteins and the host factors determines the ability of plant viruses to infect the host plants (Wang & Krishnaswamy, 2012). Given the nature of the interaction, host factors are categorized into "antiviral" and "proviral" factors. Antiviral factors are a group of host factors that inhibit the development of virus infection. The most typical antiviral host factors are conferred by resistance (*R*) genes, autophagy-related genes, ubiquitination-related genes, or mRNA decay/silencing-related genes. The antiviral host factors may interfere with the viral infection cycle, limit or inhibit viral movement, and prevent the development of infection in plants (Akhter et al., 2021; Garcia-Ruiz, 2019). In contrast, proviral host factors participate in processes essential for virus infection, that is, viral RNA translation, replication, or assembly of the virion (Garcia-Ruiz, 2019). Due to their importance in assisting viral replication and infection, proviral host factors have been targeted for developing antiviral therapy as they may be shared among related viruses.

To date, comprehensive studies on antiviral host factors have been conducted. Studies on the dominant *R* genes elucidated numerous host factors that confer resistance against pathogens, including plant viruses. The *Rsv*(s) and *Rsc*(s) in *Glycine max* that confer resistance to soybean mosaic virus (SMV) (Hajimorad et al., 2018; Tran et al., 2018; Widyasari et al., 2020), and *Pvr*(s) that confers resistance to pepper mottle virus (PepMoV) (Fang et al., 2021; Tran et al., 2015) are a few of the many dominant resistance host factors that recognize pathogen effectors and inhibit virus infection. In addition, apart from the dominant *R* genes, various independent genes are also involved in the resistance response against plant viruses. Host factors such as *GmPP2C3a*, *GmPAP2.1*, *PSaC*, and *ATPsyn-α* affect the resistance response against SMV infection by regulating innate and adaptive immune responses, including plant hormones and RNAi pathways (Bwalya et al., 2022; Seo et al., 2014; Widyasari et al., 2022).

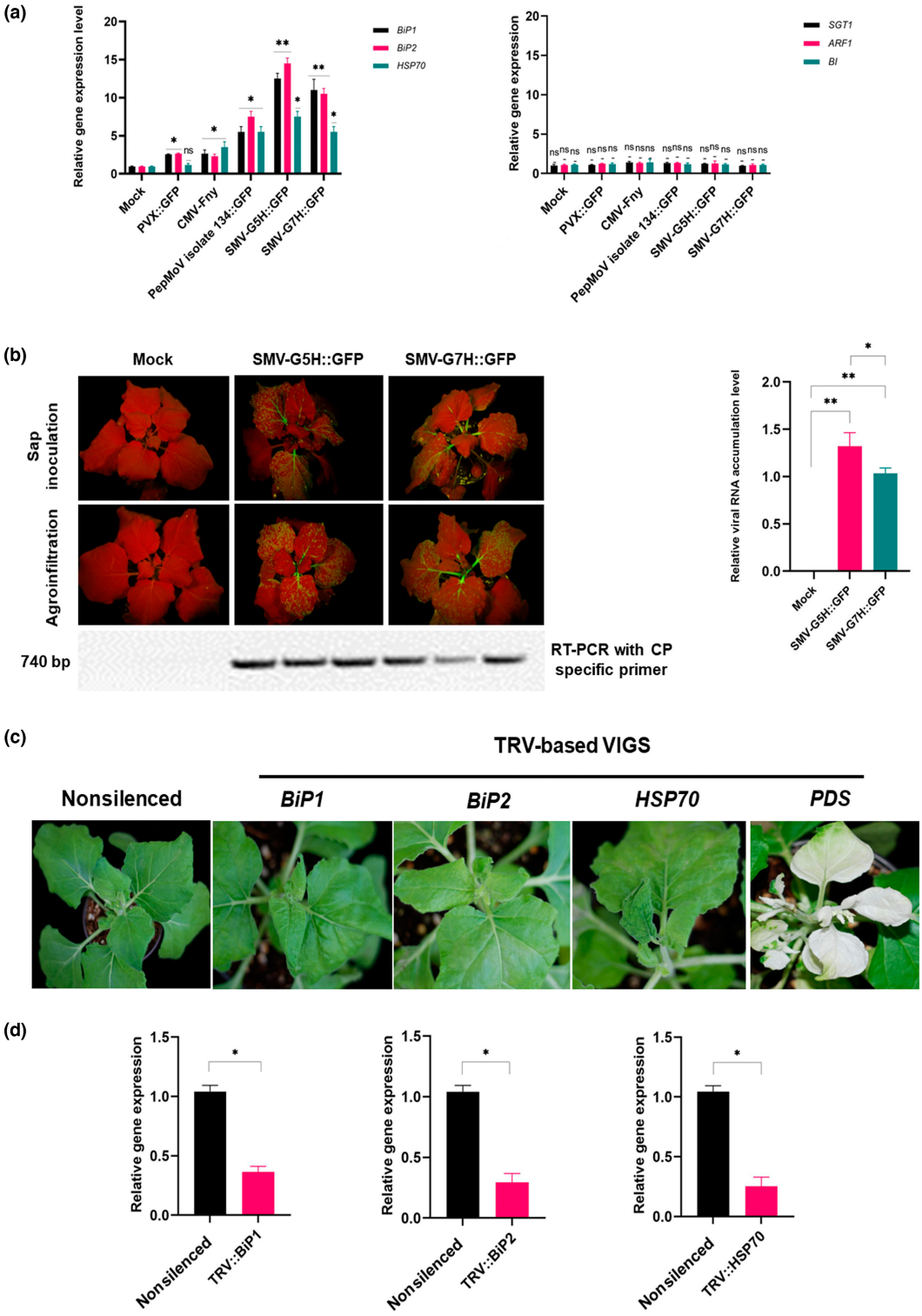
The proviral host factors translation initiation factor (eIF[iso]4 E) and DEAD-box RNA helicase RH8 (Huang et al., 2010; Lellis et al., 2002) are two crucial factors that determine the susceptibility to plant viruses. The absence of host proviral factors reduces viral replication or infection (Garcia-Ruiz, 2018; Garcia-Ruiz et al., 2018; Hashimoto et al., 2016; Hofius et al., 2007). These factors, however, do not play an essential role in the translation of plant genes or the growth and development of plants (Garcia-Ruiz, 2018). These typical host factors might be attractive targets for gene manipulation to generate a broad-spectrum viral disease-resistant cultivar. Thus, the characterization of specific proviral host factors is crucial for controlling plant virus diseases.

In this study, we characterized *Nicotiana benthamiana*'s host factors that play a crucial role in plant virus infections. We evaluated the relative transcription levels of genes encoding a suppressor of the G2 allele of skp1 (SGT1), auxin response factor 1 (ARF1), bax inhibitor (BI), binding immunoglobulins 1 and 2 (BiP1 and BiP2), and heat shock protein 70 (HSP70) in *N. benthamiana* during infection by plant viruses from the genus *Cucumovirus* (*Cucumber mosaic virus* strain Fny, CMV-Fny), *Potyvirus* (*Soybean mosaic virus* strains G5H and G7H, SMVG5H/SMV-G7H; and *Pepper mottle virus* isolate 134, PepMoV isolate 134), and *Potexvirus* (*Potato virus X*, PVX). We also evaluated the infectivity of plant viruses in the knockdown lines. Lastly, we determined the interaction between *N. benthamiana*'s host factor and the viral protein that may be crucial for virus infection.

Evaluation of the gene transcription levels by reverse transcription-quantitative PCR (RT-qPCR; see Table S1 for the gene-specific primers used for analysis) following inoculation by CMV-Fny, SMV-G5H, SMV-G7H, and PepMoV isolate 134 demonstrated a significant up-regulation of genes encoding BiP1, BiP2, and HSP70. Infection with PVX significantly induced expression of BiP1 and BiP2 but not HSP70. Expression levels of genes encoding SGT1, ARF1, and BI were not affected by infection with CMV-Fny, SMV-G5H, SMV-G7H, PepMov isolate 134, or PVX (Figure 1a).

Among the plant viruses used in this study, only SMV is known to have a relatively narrow host range, mostly restricted to two species of plants from the same genus, *G. max* and *G. soja* (Hajimorad et al., 2018). Hence *N. benthamiana* is not a natural host for SMV. Only SC7 (Gao et al., 2015) and N1 (Bao et al., 2020) strains of SMV have been reported to infect *N. benthamiana*. Interestingly, our study demonstrated the susceptibility of *N. benthamiana* to SMV strains

FIGURE 1 Expression levels of *Nicotiana benthamiana* host factors upon infection with potato virus X co-expressing GFP (PVX::GFP), cucumber mosaic virus strain Fny (CMV-Fny), pepper mottle virus isolate 134 co-expressing GFP (PepMoV isolate 134::GFP), and soybean mosaic virus strains G5H and G7H co-expressing GFP (SMV-G5H::GFP, SMV-G7H::GFP), and knockdown of *BiP1*, *BiP2*, and *HSP70*. (a) *BiP1*, *BiP2*, and *HSP70* were significantly up-regulated upon infection of CMV-Fny, PepMoV isolate 134::GFP, and SMV strains G5H or G7H. PVX infection also significantly induced up-regulation of *BiP1* and *BiP2* but not *HSP70*. Meanwhile, the expression level of *SGT1*, *ARF1*, and *BI* were not affected by viral infections. (b) The infectivity of SMV strains G5H and G7H in *N. benthamiana*. GFP expression represents SMV infection observed on the upper noninoculated leaves at 14 days after sap inoculation or agroinfiltration. The reverse transcription (RT)-PCR using SMV coat protein (CP)-specific primer indicated the presence of virus in the upper noninoculated leaves, evident by bands of 740 bp PCR product on the agarose gel. The RT-quantitative PCR analysis demonstrated a significantly higher accumulation of viral RNA in the inoculated plants than in the mock control. Plants infected by SMV strain G5H showed a relatively higher accumulation of viral RNA than those infected by SMV strain G7H. (c) The phenotype of knockdown lines by virus-induced gene silencing (VIGS). (d) The expression level of *BiP1*, *BiP2*, and *HSP70* in the knockdown lines. Values in (a), (b), and (d) are means ± *SD* from three independent experiments. Asterisks indicate significant differences and "ns" indicates the nonsignificant difference between virus-inoculated plants and mock control or between knockdown lines and nonsilenced control (*$p \leq 0.05$ or **$p \leq 0.01$, according to analysis of variance with Tukey's HSD post hoc test; Abdi & Williams, 2010)



G5H and G7H. Sap inoculation or *Agrobacterium*-mediated inoculation of SMV strains G5H and G7H co-expressing green fluorescence protein (GFP) resulted in an expression of green fluorescence on the inoculated and upper noninoculated leaves, visualizing the presence and movement of the SMV strains G5H and G7H in *N. benthamiana* (Figure 1b, upper left panel). Reverse transcription-PCR (RT-PCR) using an SMV coat protein (CP)-specific primer pair confirmed the SMV strains G5H and G7H infections in the inoculated plants, evident by the 740 base pairs (bp) product (Figure 1b, lower left panel). Consistently, evaluation of the viral RNA accumulation by RT-qPCR showed a significantly higher accumulation in the SMV strain G5H- and G7H-inoculated plants than in the mock control. However, the accumulation of viral RNA levels was observed to be higher in the plants inoculated with SMV strain G5H than G7H (Figure 1b, right panel). Hence, we chose SMV strain G5H for further analysis.

Given that infection by CMV-Fny, SMV-G5H, PepMoV or PVX induced significant expression of genes encoding BiP1, BiP2, and HSP70 (Figure 1a), we assumed that these host factors might be involved in the virus infection cycle. We therefore generated a single knockdown line for each *BiP1*, *BiP2*, and *HSP70* by a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) system and delivered them into *N. benthamiana* by *Agrobacterium*-mediated inoculation (Table S2). At 10 days postagroinfiltration (dpai) of the knockdown constructs, we observed a distinct crinkle symptom in the silenced plants. In contrast, this typical symptom was not observed in the nonsilenced control (Figure 1c). The photobleaching symptom in the phytoene desaturase (*PDS*) knockdown line was used as a phenotype control to ensure that the TRV-based VIGS system effectively down-regulates the target genes (Figure 1c). Subsequently, quantification of gene expression levels by RT-qPCR confirmed the down-regulation of *BiP1*, *BiP2*, and *HSP70* expression levels up to 65%, 73%, and 75%, respectively, in *BiP1*, *BiP2*, and *HSP70* knockdown lines compared to the nonsilenced control (Figure 1d). Interestingly, at 14 dpai of knockdown constructs, we observed a severe wilting symptom in the *HSP70* knockdown line, while neither *BiP1* nor *BiP2* knockdown lines showed any similar symptom (Figure 2a).

Binding immunoglobulin proteins (BiPs) are members of the heat shock protein 70 (HSP70) family. BiPs are relatively conserved across

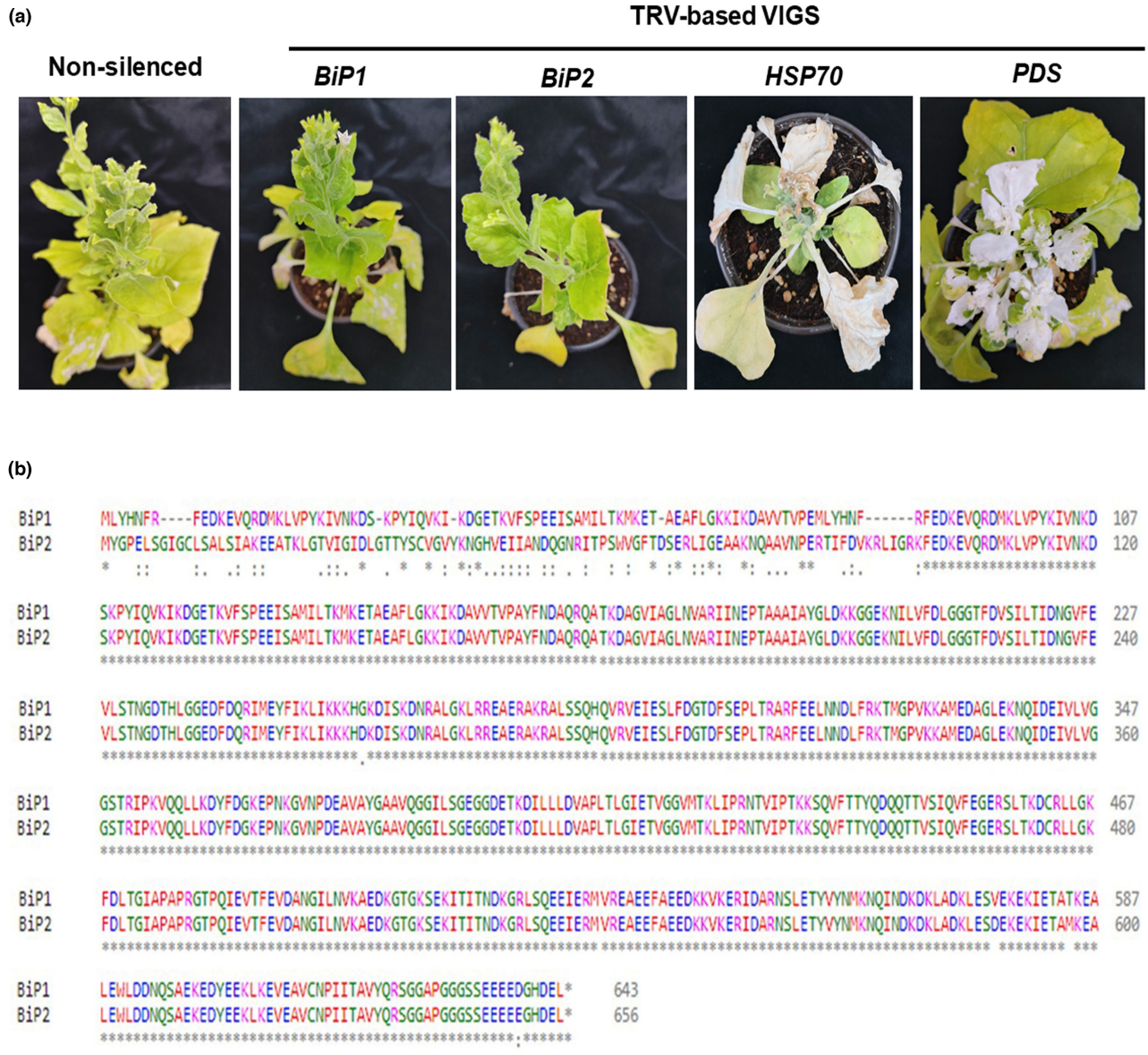


FIGURE 2 The phenotype of *Nicotiana benthamiana* at 14 days following transient knockdown of *BiP1*, *BiP2*, and *HSP70* by virus-induced gene silencing (VIGS), and the protein sequence alignment of BiP1 and BiP2. (a) Knockdown of *HSP70* caused severe wilting in *N. benthamiana*, whereas neither knockdown of *BiP1* nor of *BiP2* caused the same symptom. (b) The alignment of the BiP1 and BiP2 sequences shows differences in several amino acids, particularly in the N terminal. The different amino acids are indicated. (*) denotes a conserved sequence; (:) denotes conservative mutations; (.) denotes semiconservative mutation; () denotes nonconservative mutation

the evolutionary kingdom and among eukaryotes (Herath et al., 2020). Unlike other eukaryotes, plant BiP is encoded by multiple genes (Denecke et al., 1991; Herath et al., 2020). BiPs have numerous biological functions in plants. BiPs are primarily involved in the maturation and folding of the nonglycosylated protein (Hendershot, 2004), regulating stress transducer as part of its role in the unfolded protein response (UPR) (Bertolotti et al., 2000), and play a crucial role in the defence against various stresses such as drought stress, osmotic stress or endoplasmic reticulum (ER) stress caused by pathogen infection (Alvim et al., 2001; Reis et al., 2011; Valente et al., 2009). During plant-pathogen interactions, BiPs were reported to be targeted by PsAvh262, an effector of *Phytophthora sojae*, to suppress ER stress-triggered cell death and facilitate *P. sojae* infection in soybean (Jing et al., 2016). Overexpression of BiP in *N. benthamiana* hindered the triple gene block protein 3-induced hypersensitive response and enabled systemic movement of PVX (Ye et al., 2011).

In our study, the alignment of BiP1 and BiP2 sequences demonstrated differences in several amino acids (Figure 2b) that may lead to a diversity of natural functions of these two proteins. Because the knockdown of either *BiP1* or *BiP2* did not alter plant vigour, unlike *HSP70* (Figure 2a), whose absence had a significant impact, we assumed that the biological functions of BiP1 and BiP2 are redundant in regulating plant fitness. However, the differences in amino acids in BiP1 and BiP2 may cause differential pathogenicity-related functions.

To confirm our hypothesis, we challenge-inoculated the *BiP1* and *BiP2* knockdown lines and the ADP-ribosylation factor 1 gene (*ARF1*) knockdown line with the PVX co-expressing GFP, CMV-Fny, PepMoV isolate 134 co-expressing GFP, and SMV strain G5H co-expressing GFP. Subsequently, we observed the phenotypic symptoms and the GFP expression that visualized virus infection and movement, and quantified the viral RNA accumulation.

At 10 days after challenge inoculation by *Agrobacterium*-mediated inoculation (i.e., 20 dpai of the knockdown construct), we observed a GFP fluorescence signal that visualized PVX infection in the inoculated and upper noninoculated leaves of the nonsilenced control as well as in the *BiP1*, *BiP2*, and *ARF1* knockdown lines (Figure 3a). Similarly, following inoculation with CMV-Fny, all nonsilenced control and knockdown lines showed distinct mottling and curling symptoms (Figure 3b), whereas in the *BiP1* knockdown line inoculated with SMV strain G5H or PepMoV isolate co-expressing GFP, the fluorescence signal that visualized virus infection and movement was observed at a superficial level compared to the nonsilenced control or *ARF1* knockdown line (Figure 3c,d, TRV::BiP1 panel, white arrows indicate the GFP expression). The *BiP2* knockdown line, however, did not display any GFP fluorescence signal that visualized PepMoV or SMV infection on the inoculated or upper noninoculated leaves at 10 days after challenge inoculation (Figure 3c,d, TRV::BiP2 panel). Subsequently, evaluation of the viral RNA accumulation by RT-qPCR validated these results. No statistical difference in the viral RNA accumulation was observed in the *BiP1*, *BiP2*, or *ARF1* knockdown lines compared to the nonsilenced control at 10 days after challenge inoculation with PVX or CMV-Fny (Figure 3e). These data suggest that the absence of one of these factors did not affect the infectivity of PVX or CMV. Although a positive role of ARF1 for red clover necrotic mosaic virus infection in *N. benthamiana* has been reported (Hyodo et al., 2013), our study demonstrated that the knockdown of *ARF1* did not affect infection of PVX, CMV, PepMoV, and SMV in *N. benthamiana* (Figure 3).

Meanwhile, SMV strain G5H or PepMoV isolate 134's RNA level in *BiP1* or *BiP2* knockdown lines was significantly lower than in the nonsilenced control, with *BiP2* knockdown lines appearing to have zero RNA accumulation on leaf tissue samples (Figure 3e). Together these results suggest that *BiP1* and *BiP2* are necessary for SMV and PepMoV infection in *N. benthamiana*. Moreover, assuming that the absence of *BiP2* completely inhibits SMV and PepMoV infections in *N. benthamiana*, BiP2 may possess a more significant function in the infection of potyvirus, the largest group of plant-infecting RNA viruses. Many are widely regarded as the most economically important viral pathogens (Yang et al., 2021), making BiP2 a good candidate for gene modification to generate resistant cultivars against multiple plant viruses.

To corroborate the participation of BiP2 in the infection cycle of potyvirus, we performed an in vitro protein–protein interaction assay by yeast two-hybrid (Y2H), observing the cellular expression of BiP2 by tagging it with reporter genes for visualization, and by transiently expressing on the *N. benthamiana*, and conducted co-immunoprecipitation (Co-IP). *N. benthamiana* is a non-natural host for SMV, yet SMV strain G5H can infect this plant. We assumed that interaction between SMV proteins and the host factors is the crucial determinant for a successful SMV infection in the nonhost plants. We therefore decided to use SMV viral proteins to further investigate the interaction between potyvirus viral proteins and BiP2.

In vitro interaction demonstrated that BiP2 interacted with nuclear inclusion protein a (NIa) and nuclear inclusion protein b (NIb). The yeast colony co-expressing BiP2 and SMV protein grew better when the BiP2 was co-expressed with NIb, suggesting a stronger interaction between BiP2 and NIb (Figure 4a). Furthermore, we expressed the BiP2 tagged with mCherry and NIb tagged with GFP in the *N. benthamiana* cells and confirmed the cellular expression of either BiP2 or NIb. We observed a stronger GFP and mCherry signal in the fusion proteins (mCherry-BiP2 or GFP-NIb) than in the free GFP or mCherry (Figure 4b,c, left panel). Subsequently, we purified the plant total protein and performed western blot analysis to detect the fusion proteins. Western blot analysis confirmed the expression of BiP2 or NIb with their respective fluorescence marker in the *N. benthamiana* cell (Figure 4b,c, right panel, size of the fusion proteins is indicated). Furthermore, we also confirmed in vivo interaction between *N. benthamiana* BiP2 and SMV NIb by Co-IP analysis (Figure 4d and S1).

The NIb of potyvirus is the RNA-dependent RNA polymerase (RdRp) responsible for viral genome replication and plays a critical role in diverse virus–host interactions (Shen et al., 2020). The NIb is an active recruiter interacting with many proviral host factors to promote viral infection (Shen et al., 2020). Studies on the NIb of turnip mosaic virus (TuMV) demonstrated the interaction of NIb

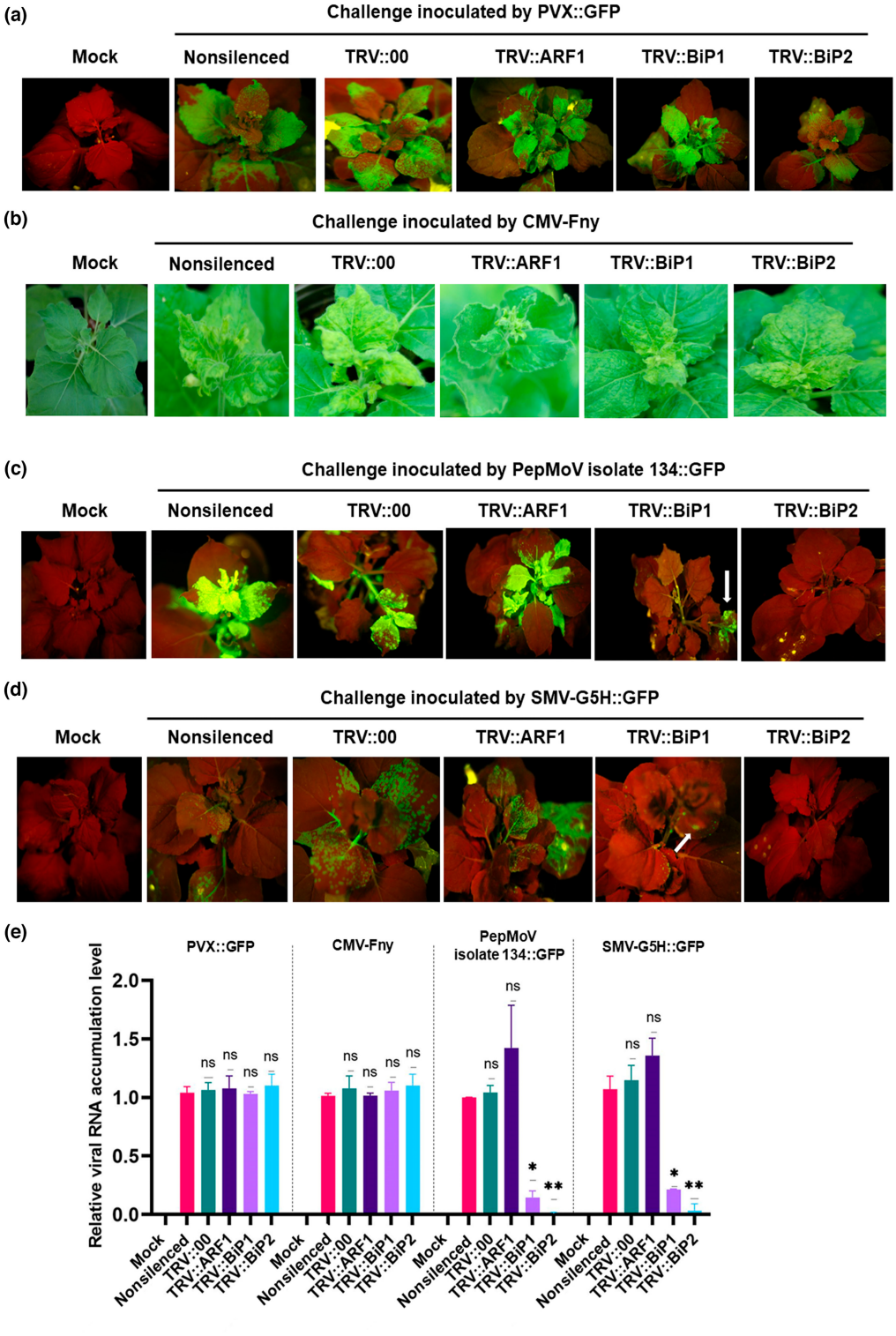


FIGURE 3 Symptoms and viral RNA accumulation level in the nonsilenced *Nicotiana benthamiana* and in plants knocked down by TRV::00, TRV::ARF1, TRV::BiP1, and TRV::BiP2, which were inoculated with PVX co-expressing green fluorescent protein (GFP), CMV-Fny, PepMoV isolate 134 co-expressing GFP, and SMV co-expressing GFP. (a) A GFP signal visualizing PVX infection in all inoculated plants. (b) All plants inoculated by CMV-Fny developed a distinct mottling symptom. (c) and (d) A GFP signal visualizing infection of PepMoV isolate 134 and SMV strain G5H at moderate intensity in *BiP1* but not in the *BiP2* knockdown lines. A white arrow points to the GFP signal. (e) Accumulation of viral RNA in the nonsilenced and knockdown lines infected by plant viruses. Values are means ± *SD* from three independent experiments. Asterisks indicate significant differences and "ns" indicates the nonsignificant difference between nonsilenced control and knockdown lines (*$p \leq 0.05$ or **$p \leq 0.01$, according to analysis of variance with Tukey's HSD post hoc test; Abdi & Williams, 2010)

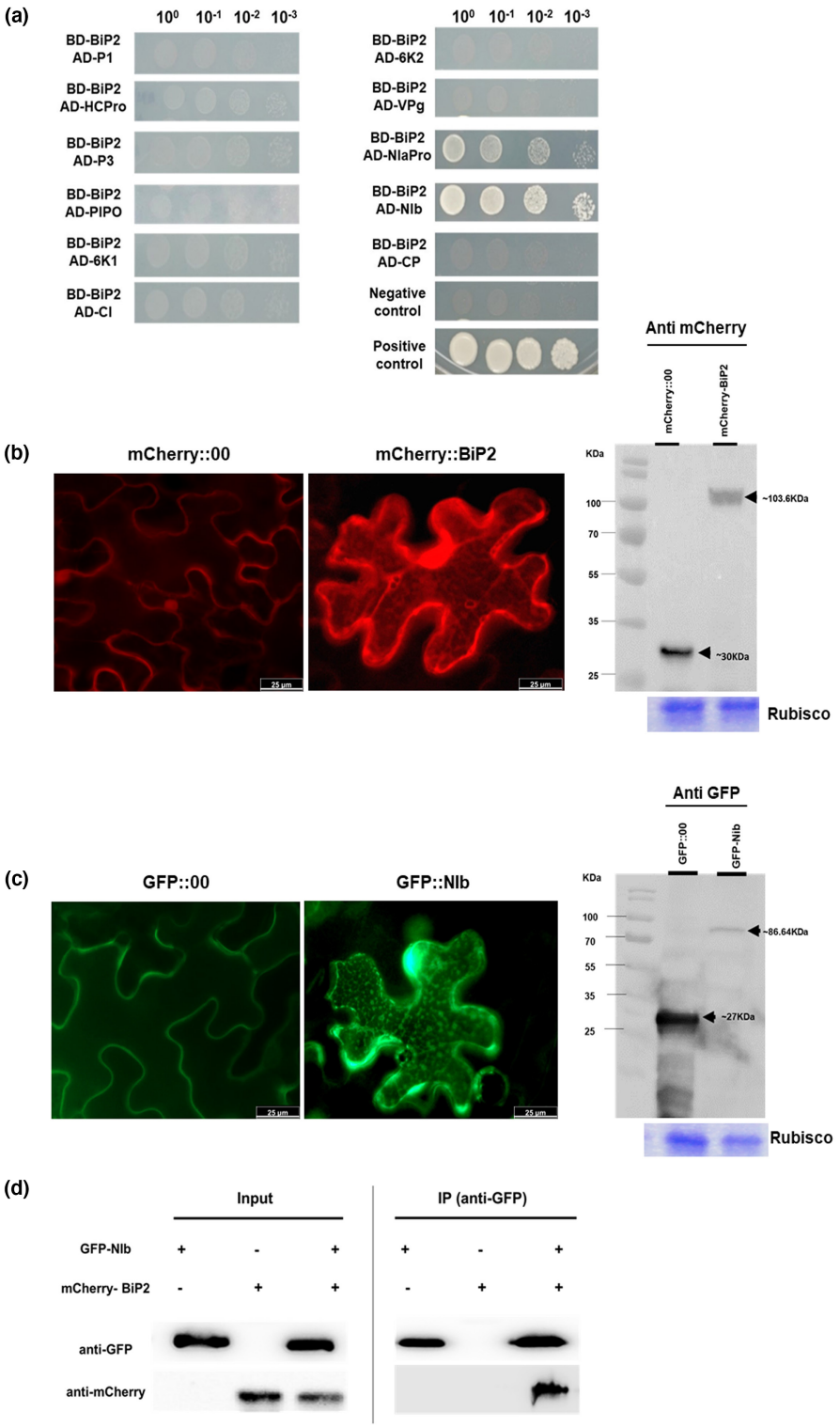


FIGURE 4 Cellular expression and interaction of BiP2 and NIb. (a) Yeast two-hybrid analysis of BiP2 and 11 viral proteins of SMV strain G5H. (b) Expression of BiP2 tagged with mCherry in the *Nicotiana benthamiana* cell and the western blot result with the size of the fusion protein as indicated. (c) The expression of NIb tagged with green fluorescent protein (GFP) in the *N. benthamiana* cell and the western blot result showing the size of the fusion protein as indicated. (d) Co-immunoprecipitation analysis demonstrated a direct interaction between *N. benthamiana* BiP2 and SMV NIb

with the heat shock cognate protein 70-3 (Hsc70-3) and poly(A)-binding protein (PABP) that promotes viral infection. The association of Hsc70-3 and NIb could occur in membrane-derived replication complexes (Dufresne, Thivierge, et al., 2008; Dufresne, Ubalijoro, et al., 2008). Correspondingly, a study on the NIb of another potyvirus, potato virus Y strain necrotic tuber necrosis (PVY^{NTN}), demonstrated its interaction with Hsc70, resulting in susceptibility to PVY^{NTN} (Kozieł et al., 2021). Given that interaction between NIb and host factors primarily results in infection, there is a high possibility that NIb is recruiting these host factors into the viral replication complex (VRC) for virus multiplication (Shen et al., 2020). Viral 6K2 protein facilitates the development of VRCs by remodelling the ER for this purpose (Wei et al., 2010). The recruitment of NIb into the VRC may not be through direct interaction with 6K2 but most probably via its interaction with the VPg domain of 6K2-VPg-NIaPro (Li et al., 1997, 2020). Hence, SMV NIb, which interacts with BiP2 of

N. benthamiana, may also be recruited into the VRC via interaction with the VPg domain of 6K2-VPg-NIaPro to initiate replication and translation. Nevertheless, our inability to incorporate the ER marker for visualization and to demonstrate the interaction between the NIb-BiP2 complex with the VPg domain of 6K2-VPg-NIaPro is the main limitation for determining the underlying recruitment mechanism of NIb-BiP2 complex to the VRC.

Lastly, given that BiPs are highly conserved in many species, we extended our study by characterizing the homology and expression level of gene encoding BiP2 in *G. max*, a natural host of SMV. A phylogenetic gene sequence analysis demonstrated the presence of a gene encoding BiP2 in *G. max* (Figure S2). Homologue genes may share many similarities in biological properties and functions (Brigandt & Griffiths, 2007), which in our study may be related to the BiP2 functions in the potyviruses, particularly the SMV infection cycle. We further confirmed this causality by RT-qPCR and validated the expression level of *BiP2* in *G. max* following infection of SMV strains G5H and G7H. The result of RT-qPCR analysis demonstrated a significant up-regulation of *BiP2* in SMV-infected *G. max* compared to the mock control (Figure S3), indicating that *BiP2* in *G. max* is also regulated by potyvirus infection.

In summary, our study provides information on the proviral host factors (BiP2) that play a crucial role in potyvirus infection; hence BiP2 may be a promising candidate for gene manipulation to generate a broad-spectrum viral disease-resistant cultivar. Nevertheless, further studies are needed to elucidate the mechanism underlying the recruitment of the BiP2-NIb complex into the VRC and its contribution to viral multiplication.

AUTHOR CONTRIBUTIONS

K.W., J.B., and K-H.K conceived the ideas and designed the experiments. K.W. and J.B. carried out the experiments. K.W. analysed the data and wrote the manuscript. K.-H.K. edited the manuscript and acquired the funding. All authors read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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