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콩 모자이크 바이러스와 기주 인자 간 상호작용 기작의 구명 : 콩 모자이크 바이러스의 감염에 대한 GmPAP 2.1과 HSP 70 단백질의 서로 다른 역할

Elucidating the complex interplay between host factors and soybean mosaic virus (SMV): Study on the GmPAP 2.1 and HSP 70 proteins that play antagonistic roles in the development of SMV infection.

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Elucidating the complex interplay between host factors and soybean mosaic virus (SMV): Study on the GmPAP 2.1 and HSP 70 proteins that play antagonistic roles in the development of SMV infection.

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Elucidating the complex interplay between host factors and soybean mosaic virus (SMV): Study on the GmPAP 2.1 and HSP70 proteins that play antagonistic roles in the development of SMV infection.

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ABSTRACT

Interaction between plant pathogens and their host plants is a complex interplay that involves numerous host factors. These interactions, however, will determine the fate of either successful infection in plants or the development of resistance response. Hence, understanding the interaction between invading virus and host factors may give us deep insight into the virus infection processes. In this study we disclosure interaction between soybean mosaic virus (SMV) and two soybean host factors that lead to two antagonistic developments of SMV infection. We found that overexpression of GmPAP2.1 from L29 conferred SMV resistance to a susceptible cultivar, Lee 74. We determined that

GmPAP2.1 interacted with the SMV protein P1 in the chloroplasts, resulting in the up-regulation of the *ICS1* gene, which in turn promoted the pathogen-induced salicylic acid (SA) pathway. On the other hand, SMV infections in *Nicotiana benthamiana* provoke upregulation of genes that encodes BIPs, members of the HSP70 family protein. Subcellular co-localization and yeast two hybrids analyses demonstrated an interaction between NIb protein and BIP 2 in the ER. Transient knock-down of this gene impairs SMV infection, suggesting the importance of BIP 2 protein in the SMV infection in *N.benthamiana*. Furthermore, I also observed the upregulation of a gene that encodes BIP 2 protein in Glycine max. Together, my study provides new information about host factors that play antagonistic roles in the SMV infection, and subsequently, these finding may extent the possibility to assist management of SMV incidence in the future through modification of host factor that involves in the SMV infection.

Keyword: soybean mosaic virus, *Glycine max*, *Nicotiana benthamiana*, host factors, interaction, resistance

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Soybean resistance to soybean mosaic virus

[a] This chapter is a slightly modified version of paper that previously has been published in PLANTS volume 9 issue 2, 10.3390/plants9020219

ABSTRACT

Soybean mosaic virus (SMV) distributed in all soybean-growing areas in the world and causes huge losses in soybean yields and seed quality. Molecular interactions between SMV effector proteins and the soybean resistance (R) protein, if present, determine the development of resistance/disease in soybean plants. R-protein in resistant soybean perceives a specific SMV effector, which triggers either the extreme silent resistance or the typical resistance manifested by hypersensitive responses and induction of salicylic acid and reactive oxygen species. Here, I consider the major advances that have been made in understanding the soybean–SMV arms race. I also focus on dissecting mechanisms SMV employs to establish infection and how soybean perceives and then responds to SMV attack. In addition, progresses on soybean *R*-genes studies, as well as those addressing independent resistance genes, are also addressed.

Keywords: extreme resistance; *R*-gene resistance; soybean; soybean mosaic virus

INTRODUCTION

Soybean mosaic virus (SMV), from the genus *Potyvirus* and the family *Potiviridae*, causes soybean mosaic disease, a disease that greatly reduces soybean production worldwide (Hartman & Hill, 2010; Hartman et al., 2011a). SMV has a very narrow host range which is limited to six plant families: *Fabaceae, Amaranthaceae, Chenopodiaceae, Passifloraceae*, *Schrophulariaceae*, and *Solanaceae*. The most commonly infected hosts are *Glycine soja* (wild soybean) and *G. max* (cultivated soybean) (Hajimorad et al., 2018b; Hill & Whitham, 2014). Management of SMV is limited to the use of good agricultural practices and the development of resistant cultivars via breeding and genetic engineering (Galvez et al., 2014). Several SMV strains, however, have evolved the ability to avoid recognition by the plant R-protein and to thereby establish infections which lead to the emergence of resistance breaking SMV strains (Choi et al., 2005b; Koo et al., 2005b). Hence, improving the understanding of how soybean perceives and responds to SMV infection will help the development of molecular breeding towards broad-spectrum resistance against SMV.

Soybean and SMV interact in complex ways during each step of infection. SMV passively enters plant cells through natural openings or through

physical wounds caused by environmental factors or insect vectors (Kang et al., 2005). If the host is unable to recognize the SMV effector(s), a compatible interaction is established. The severity of the resulting disease depends on the ability of the virus to hijack host proteins and suppress immune responses (Calvo et al., 2014).

According to the mode of interaction between plant and viruses, resistance is often classified into recessive resistance and dominant resistance. Recessive resistance is established upon the impairment of a host factor required for virus replication, or negatively involved in resistance (Hashimoto et al., 2016). In contrast, dominant resistance which leads to incompatible interaction is triggered upon the recognition of viral effector by the host resistance (R) protein (Galvez et al., 2014; Kang et al., 2005). The incompatible interaction between soybean and SMV is characterized by the induction of salicylic acid (SA), the development of a hypersensitive response (HR), and a burst in the production of reactive oxygen species (ROS). These lead to the death of the infected cells, and trap the virus at the point of infection (Baebler et al., 2014; Calvo et al., 2014; Hajimorad & Hill, 2001; Hajimorad et al., 2005). SA is a hallmark in many incompatible interactions, including *Rsv1*-mediated resistance against the SMV-N avirulent strain (Alazem, 2015; Baebler et al., 2014; Zhang et al., 2012). Interestingly, abscisic acid (ABA), which

antagonizes the SA effect, appears to play a critical role in the incompatible interaction between the resistance gene *Rsv3* and the avirulent strain SMV-G5H (Alazem et al., 2018a; Alazem et al., 2019). Both SA and ABA have been reported to positively regulate plant resistance against several viruses [in both compatible and incompatible interactions], but some viruses are able to reverse the defensive effects of ABA (Alazem et al., 2017; Alazem & Lin, 2017; Alazem et al., 2019; Xie et al., 2018).

It is well-known that plants have evolved defense mechanisms against viruses and other pathogens. Researchers have made substantial progress in understanding the ability of plants to defend against viral pathogens (Chen et al., 2015; Luan et al., 2019; Xun et al., 2019). In soybean, three independent loci (*Rsv1, Rsv3,* and *Rsv4*) have been characterized to confer resistance against SMV strains G1–G7 (Maroof et al., 2008; Shakiba et al., 2012b) and other resistance loci (*R*-genes: *Rsc4, Rsc5, Rsc7, Rsc8, Rsc15,* and *Rsc20*) where characterized in China to confer resistance against SMV strains SC1–SC21 (Karthikeyan et al., 2017a; Karthikeyan et al., 2018a; Rui et al., 2017b; Wang et al., 2017; Wang, et al., 2011; Wang, et al., 2011b).

BIOLOGICAL PROPERTIES AND TRANSMISSION OF SMV

I. SMV genome and gene function

SMV has been grouped into seven strains (G1 to G7) based on its virulence to soybean lines cultivated in the United States (Cho & Goodman, 1979), and into 21 strains (SC1 to SC21) based on the Chinese identification system (Liang et al., 2010; Wang et al., 2013). The SMV genome consists of a single-stranded positive-sense RNA, which is approximately 10 kb long and associated with genome-linked viral protein (VPg) bound to the 5' end and the poly (A) tail at the 3' end of the viral genome. Both the RNA and VPg are encapsidated in rod-shaped coat protein (CP) (Hajimorad et al., 2018b; Riechmann et al., 1992). The genome encodes one large ORF, which is translated into a large polyprotein and subsequently undergoes a proteolytic reaction yielding 10 different functional proteins. As a result of a frameshift on the P3 cistron, the SMV genome also includes a small ORF that encodes an 11^{th} protein with a size of 25 kDa (Olspert et al., 2015). These 11 proteins are P1, HC-Pro, P3, PIPO (a product of slippage in the P3 coding sequence), 6K1, CI, 6K2, VPg, NIa-Pro, NIb, and CP (Table 1) (Chung et al., 2008; Olspert et al., 2015; Revers & García, 2015).

Table 1 Summary of the biological functions of SMV proteins.

Protein	Function for virus	Function for plant
P1	Protease, Viral host range	
HC-Pro	Long-distance movement, a 'bridge' between virion particles and aphid stylets in aphid transmission, suppression of host defense (RNA silencing).	Virulence determinant
P3	Targets host elongation factors 1A (eEF1A) to facilitate SMV replication	Effector of *Rsv1*
PIPO	Movement	
6K1	Cell-to-cell movement	
CI	Required for genome replication and movement (cell-to-cell or long-distance movement)	Effector of *Rsv3*
6K2	Formation of the virus replication complex	
VPg	Binds specifically to eIF4E to initiate polyprotein translation	
NIa-Pro	Proteinase	
Nib	The catalytic subunit of RdRp	
CP	A 'bridge' between virion particles and aphid stylets in aphid transmission, cell-to-cell movement, virus assembly.	

II. Biological and molecular properties of SMV transmission

SMV replicates in the cytoplasm in virus replication complexes (VRCs) which are associated with endoplasmic reticulum (ER) (Wei, et al., 2010). P3 recruits the host elongation factor 1A (eEF1A), to initiate unfolded protein response (UPR), an adaptive response involves the accumulation of unfolded proteins at the ER, which in turn facilitates SMV replication (Luan et al., 2016). VPg protein binds with eIF4E to initiate translation of the polyprotein, which is subsequently cleaved by viral proteases to produce 11 distinct functional proteins (Hajimorad et al., 2018b; Helm et al., 2019; Michon et al., 2006).

Systemic infection by most plant viruses, including SMV, comprises two processes: cell-to-cell movement through plasmodesmata (PDs), and long-distance trafficking through the vascular system. PDs are essential for the intracellular trafficking of molecules required for plant life, and plant viruses have evolved to manipulate this communication system to facilitate intercellular movement (Reagan & Burch-Smith, 2019). The SMV MP and CP+HC-Pro complex increases PD size exclusion limits to facilitate the movement of virions into neighboring cells (Allie et al., 2014; Otulak-Kozieł et al., 2018). In the case of turnip mosaic virus, movement is also assisted by PIPO, a protein required for virus movement (Wei, et al., 2010). PIPO directs the CI protein to

the PD where it forms a PIPO-CI complex. This complex coordinates the formation of a PD-associated structure, which facilitates the intercellular movement of the virion in the infected plants (Vijayapalani et al., 2012; Wei, et al., 2010). In addition, the 6K1 protein localizes to the cell periphery, where it is thought to have an essential function in cell-to-cell movement (Hong et al., 2007). The viral genome is transported from the epidermal to mesophyll cells through PDs; once the viral genome reaches the vascular bundles, long-distance trafficking of the virus is initiated (Figure 1).

SMV is seed- and aphid- transmitted virus, and aphids uptake SMV in a non-persistent manner (Nachappa et al., 2016; Seo et al., 2010). Aphid transmission depends on the interaction between HC-Pro and CP proteins. The presence of a DAG sequence in the CP facilitates the transient binding of the CP to HC-pro, and is essential for the binding of virus particles to the aphid stylet and thus for aphid transmission (Jossey et al., 2013; Seo et al., 2010).

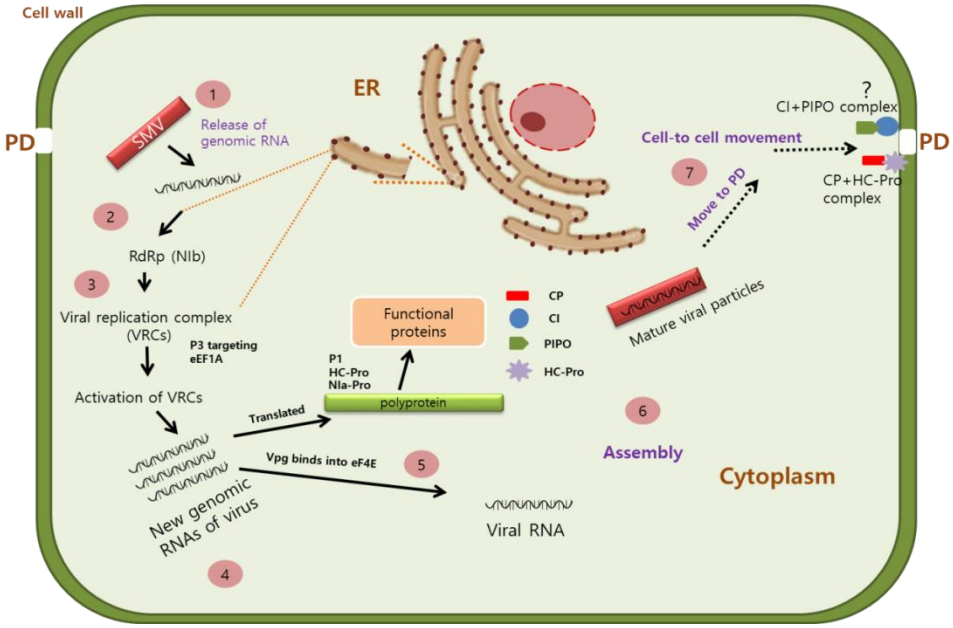

Fig. 1. Replication and movement of soybean mosaic virus (SMV) within the cell. SMV enters the plant cell through natural openings such as the plasmodesmata (PD) or openings on the plant surface resulting from mechanical injury. Upon SMV entry, the viral genomic RNA is released, and translated. Following translation of the viral proteins, virus particles assemble, and the new virus progeny move to neighboring cells. Virus movement is assisted by several functional proteins. The coat protein (CP) protects the genomic RNA, prevents degradation of viruses or virus components by host factors, and delivers the genomic RNA to PD. at PD, the proteins CI and PIPO form a CI-PIPO complex to coordinate the formation of the PD-associated structure which facilitates the intracellular movement of the virus.

RESISTANCE GENES (*R*-GENES): SOYBEAN RESPONSE TO SMV INFECTION

I. NLR Gene Family-Mediated Resistance to SMV

Host resistance proteins with nucleotide-binding (NB) domains and leucine-rich repeats (LRR), shortly termed as (NLRs), represent a major class of plant immune receptors that greatly affect host–pathogen interactions (Noman et al., 2019; Wu et al., 2017). Upon perception of pathogen effectors, NLRs trigger a cascade of downstream defense events leading to the induction of resistance against the invading viruses (Cesari, 2018). NLRs may represent the evolution of multifunctional single receptors, which combine sensor activity (helper) and immune signaling (executor) in a single protein, into networks of functionally interconnected receptor pairs (Adachi et al., 2019). During the perception phase, NLRs sense viral effectors directly or indirectly, and triggers an HR in the host (Adachi et al., 2019). Most R-proteins have NLR domains located in their N-termini. NLRs are divided into two subfamilies: one with a Toll/interleukin-1 receptor (TIR) domain, and the other with a coiled-coil (CC) structure (McHale et al., 2006). TIR motifs of R-proteins are often found in dicotyledonous plants (Deslandes et al., 2002; Meyers et al., 2002). A comprehensive study of NLR-type *R*-genes led to the identification and

characterization of two groups pf dominant *R*-genes in soybean which confer resistance against SMV; 1) *Rsv* genes which confer resistance to strains G1 to G7 in the United States (Khatabi et al., 2012; Liu et al., 2016), and 2) *Rsc* genes which confer resistance to SMV strains SC1 to SC22 in China (Liang et al., 2010; Wang et al., 2014; Zhan et al., 2006).

II. *Rsv* Genes

Rsv1, Rsv3, Rsv4, and *Rsv5* are four loci that confer resistance to different SMV strains. *Rsv1* is a highly complex locus with multiple alleles mapped to molecular linkage group (MLG) F. The dominant *Rsv1* locus is mapped to chromosome 13. Candidate genes encoded in the cultivar PI 96983 were identified as a cluster of nucleotide-binding leucine-rich repeat (NB-LRR)-type of *R*-genes (Hayes et al., 2004). *Rsv1* confers resistance to SMV strains G1 to G6 but not to G7 (Shi et al., 2009). Phenotypes of *Rsv1*-mediated resistance against SMV strains are diverse and include extreme resistance (ER) against SMV strains G1 to G6, lethal hypersensitive response (LSHR) against SMV-G7 (Hajimorad et al., 2006), and HR occurring on the stem, petioles, and leaf veins of plants inoculated with G2 (Hajimorad & Hill, 2001).

Rsv1-mediated ER against most SMV strains requires multiple defense genes including those involved in the SA and JA pathways, and may also involve specific WRKY transcription factors (Wang et al., 2014; Zhang et al., 2012). Silencing soybean orthologs of the SA-related genes *GmEDR1*, *GmEDS1*, and *GmPAD4*, and the JA-related gene *GmJAR1* in the SMV-resistant soybean line L78-379 resulted in symptoms that were similar to those in a susceptible control cultivar (Williams 82) in response to inoculation with SMV-G2 (Zhang et al., 2012). In another study, double silencing of *GmEDS1a/GmEDS1b* or single silencing of *GmPAD4* reduced pathogen-inducible SA accumulation, which further enhanced soybean susceptibility to SMV-G5 and thereby indicated the importance of SA in *Rsv1*-resistance against SMV-G5 (Wang et al., 2014). In addition, silencing *GmHSP90* severely stunted plants and reduced the replication and movement of SMV-G2 (Zhang et al., 2012). This suggests that the chaperone HSP90 is required for Rsv1-mediated ER in response to G2, an avirulent strain of SMV (Hajimorad et al., 2018b).

Many WRKY transcription factors regulate the transcriptional reprogramming associated with plant immune responses and plant development (Eulgem & Somssich, 2007; Pandey et al., 2010). Several reports suggest that SA-related WRKYs are actively involved in Rsv1-mediated resistance against SMV-G2. For example, silencing the SA-regulated WRKY6 and WRKY30 in

the soybean line L78-379 compromised the Rsv1-mediated resistance against SMV-G2 in soybean line (Choi et al., 2015; Robatzek & Somssich, 2002; Zhang et al., 2012)

The P3 protein is the effector of *Rsv1*-mediated resistance, and the amino acids 823, 953, and 1112 are important Rsv1 perception of P3, and thus for the subsequent induction of LSHR (Hajimorad et al., 2006; Hajimorad et al., 2005) (Figure 2). Replacement of HC-Pro and/or P3 of avirulent strains with HC-Pro and/or P3 from virulent strains (SMV-G7 or SMV-G7d) changed the avirulent strains into virulent strains (Wen et al., 2013), suggesting that HC-Pro is also an effector for *Rsv1*-mediated resistance.

Rsv3 is mapped to a locus between the markers A519F/R and M3Satt on chromosome 14 in the soybean molecular linkage group B2 (Suh et al., 2011). Further investigation revealed that the *Rsv3* locus contains a family of closely related proteins with a CC motif and an LRR domain (CC-NB-LRR), suggesting that *Rsv3* encodes a member of the NLR family (Suh et al., 2011). Analysis of the soybean genome revealed that five different NBS-LRRs sequences are exist between the flanking marker (Tran et al., 2018). Unlike *Rsv1*, which confers resistance to a broad spectrum of SMV strains, *Rsv3* is strain-specific resistant gene (Tran et al., 2018). *Rsv3* confers ER only to SMV strains G5, G6, and G7, and G5H (Gunduz, 2000; Jeong et al., 2002a; Seo et al.,

2009). However, *Rsv3* induces a necrosis and mosaic symptoms depending in the infecting strain (G1 to G4), and induces systemic mosaic symptoms upon the infection with G7H (Seo et al., 2009). Analyses of chimeras that were constructed by exchanging fragments between avirulent SMV-G7 and virulent SMV-N showed that both the N and C terminal regions of the CI cistron are required for Rsv3-mediated ER (Zhang et al., 2009). In a different study, a single amino acid substitution in the CI region between G7H and G5H was responsible for the ER induction in response to G5H infection. (Seo et al., 2009) (Figure 2).

The molecular signaling involved in the Rsv3-mediated ER was elucidated using the *Rsv3*-harbouring L29 plants. Infection with G5H allows Rsv3 to recognize the CI protein which induces several genes in the ABA pathway, including the negative regulator *PP2C3a* (Alazem et al., 2018a; Seo et al., 2014). Expression of *PP2C3a* induces callose accumulation, and thus restricts G5H movement at the infected points (Seo et al., 2014). Analysis of RNA sequencing data also suggested that the *Rsv3*-mediated ER against SMV-G5H involves, the antiviral RNA silencing pathway and autophagy. Besides, reduction in the expression of many genes in the jasmonic acid pathway and *WRKY* transcription factors were also observed following G5H infection on L29 plants. Interestingly, ABA can also induce resistance L29 plants against the

G7H virulent strain by enhancing callous accumulation and increasing the expression of several genes involved in the antiviral RNA silencing pathway (Alazem et al., 2018a; Alazem et al., 2019). Future research addressing the localization of Rsv3, factors associated with Rsv3, and downstream defense signaling pathways would help us better understand the molecular mechanisms underlying *Rsv3*-mediated resistance.

The *Rsv4* locus is flanked by the microsatellite markers (SSRs) Satt542 (4.7 cM) and Satt558 (7.8 cM) (Hayes et al., 2000). Using whole genome sequencing of D26 (which carries the *Rsv4 gene* crossed with Lee 68 (an *rsv*-null cultivar), and of V94-5152 (*Rsv4*) crossed with Lee 68 (*rsv*), it has been determined that *Rsv4* is localized in the 1.3 cM region on chromosome 2 (Maroof et al., 2010). This region does not have any NLR gene, however, several genes carried in the region encode for transcription factors (Maroof et al., 2010). Rsv4 confers resistance to strain G1 to G7 (Gunduz et al., 2004). In G2 strain, a single amino acid substitution (Q1033K) in P3 protein enabled the mutant to overcome Rsv4 resistance in the soybean cultivar V94-5152 (Chowda-Reddy et al., 2011). Sequence analysis of new variants of *Rsv4*-resistance-breaking isolates revealed that these isolates contained either the Q1033K mutation or a G1054R substitution in their P3 protein (Chowda-Reddy et al., 2011). The combination of Q1033K and G1054R enhanced SMV

movement and symptom severity in the soybean PI 88788 (*Rsv4*) (Khatabi et al., 2012). These results suggest that SMV virulence determinants in *Rsv4* cultivars are located on P3, and that Q1033K or G1054R substitution is sufficient to increase SMV virulence (Chowda-Reddy et al., 2011; Khatabi et al., 2012; Wang et al., 2015).

The strength of *Rsv4*-mediated resistance and the nature of the associated phenotypes differ between two cultivars carrying the *Rsv4* gene (V94-5152 and PI 88788) (Gunduz et al., 2004; Khatabi et al., 2012). While SMV-N accumulated in the inoculated leaves of both cultivars, infection was much less severe in V94-5152 than in PI 88788. These results indicate that *Rsv4*-mediated resistance is affected by the genetic background of the cultivar, i.e., the local and systemic movement of SMV depends on the *Rsv4* cultivar (Khatabi et al., 2012).

Given that *Rsv4* does not encode NLR genes, and that *Rsv4*-mediated resistance is quite different from *Rsv1*- or *Rsv3*- mediated resistance, it was proposed that *Rsv4* belongs to a new class of resistance genes (Maroof et al., 2010). A recent study showed that *Rsv4* encodes an RNase-H family protein with dsRNA-degrading activity, and interacts with the P3 protein of SMV to promote the fusion of dsRNAses with host factors involved in virus replication. This fusion result in the degradation of the viral dsRNAs (Ishibashi et al., 2019).

A study on the *Rsv1* locus revealed that *Rsv1* and *Rsv1-y* are separated by 2.2 cM on chromosome 13 of soybean cultivar York (Klepadlo et al., 2017). This substantial separation suggested renaming *Rsv1-y* to become *Rsv5*. *Rsv1-y* confers resistance to G1 but not to G7 (Zheng et al., 2005). The cultivar York was developed from a cross between Dorman (developed from Dunfield and Arksoy) and Hood. Similar to York, Dorman and Arksoy are resistant to G1 but not to G7, suggesting that *Rsv1-y* in York came from Arksoy (Zheng et al., 2005). Pedigree analysis of 18 other soybean genotypes derived from Arksoy showed that Riple, Calhoun, and Musen have *Rsv1-y*-mediated resistance (Zheng et al., 2005). The mechanism underlying *Rsv1-y* (or *Rsv5*)-mediated resistance remains unknown.

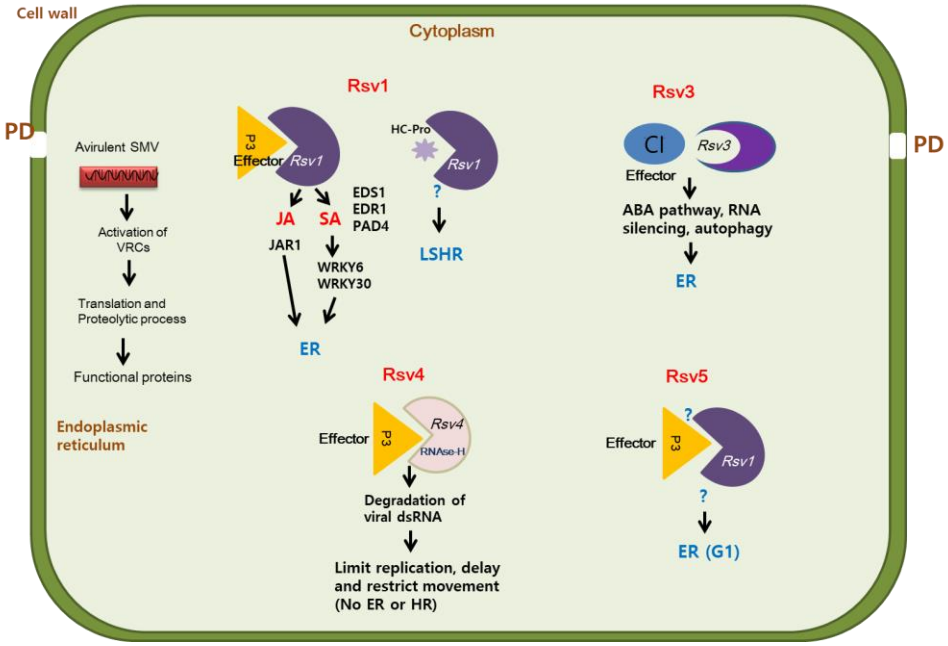

Fig. 2. Rsv-mediated perception and resistance against SMV. Rsv1 recognizes the effectors P3 and/or HC-Pro protein; recognition of HC-Pro induces a lethal systemic hypersensitive response (LSHR), and recognition of P3 induces several host factors including *HSP90*, *EDS1*, *EDR1*, *WRKY6*, and *WRKY30*, which contribute in extreme resistance (ER) through the salicylic acid (SA) and jasmonic acid pathways. Rsv3 recognizes the CI protein and thereby induces ER where abscisic acid (ABA), and antiviral RNA silencing pathway and autophagy are triggered following infection. Rsv4 recognizes P3, which encodes dsRNAase, and targets the viral dsRNA in the replication complex leading to its degradation. The effector for Rsv5 is unknown, but the recognition results in ER in response to SMV-G1.

Table 2 Summary of *R* genes conditioning resistance to SMV

***R* gene**	**SMV strain**	**Cultivar**	**Location**	**Effector**	**Type of *R* gene**
Rsv1	G1-G6	Kwanggyo Marshall Odgen PI96983 PI507389 Raiden Suweon97 Kosuzu Susumaru PI39887 Jitsuka Clifford Tousan65 Corcisa PI61944 PI61947	Chromosome 13	P3, HC-pro	NB-LRR-type of *R*-genes
Rsv3	G5,G6, G7	Columbia Hardee Tosan140 PI 339870 PI399091 L29	Chromosome 14	CI	CC-NB-LRR type or R-gene
Rsv4	G1-G7	PI486355 V94-5152 P188788 Haman Ilpumgeomjeong KAERI-GNT-220-7 PI 398593 PI438307 Rhosa Beeson	Chromosome 2	P3	Non-NLR genes (RNase-H family protein)
Rsv5	G1	York Dorman Arksoy Riple Calhoun Musen	Chromosome 13	Possibly P3	unknown

III. *Rsc* Genes

The nation-wide SMV strain identification system in China includes 22 SMV strains, designated as SC1–SC22. These strains are identified based on their response to 10 dominant soybean cultivars that are distributed in different areas in China (Karthikeyan et al., 2018a; Liang et al., 2010). Genes conferring resistance to SC strains are designated as *Rsc* resistance genes, and mapped to the same chromosomes as *Rsv* genes (chromosome 13, 14, and 2) (Table 3) (Hajimorad et al., 2018b; Liang et al., 2010) .

Apart from those genes indicated in table 3, a novel locus discovered on chromosome 6 was found responsible for SMV-resistance in the soybean cultivar RN-9 (Rui et al., 2017b). The new locus was designated *Rsc15* and was mapped to a 14.6-cM region that is flanked by two SSR markers: SSR_06_17 and BARCSOYSSR_06_0835 (Rui et al., 2017b). The expression of *Rsc15* during the early stages of SMV-SC15 infection in RN-9 was highly correlated with hydrogen peroxide (H_2O_2) levels and peroxidase (POD) activity (Rui et al., 2017b). *Glyma06g182600* was designated as *GmPEX14* and proposed as the strongest candidate gene of *Rsc15.* It encodes a peroxisomal membrane anchor protein and has a polymorphism in the DNA/cDNA sequence alignments. Infection by SC15 increased the expression of *GmPEX14*, and induced the

H_2O_2 burst in the resistant cultivar RN-9 (Rui et al., 2017b). This suggests that peroxidases are probably involved in *Rsc15*-mediated resistance to SC15.

In addition to single dominant resistance genes, a combination of SMV resistance genes has also been reported in China. Crosses between soybean cultivars Qihuang1 x Kefeng 1 and Dabaima x Nannong 1138-2 resulted in plants carrying *Rsc4*, *Rsc8*, and *Rsc14Q* genes, which confer resistance to 21 strains of SMV in China (Wang et al., 2017). In addition, pyramiding has been used to obtain soybean lines with combinations of resistance genes. Gene pyramiding in Essex cultivar was used to generate *Rsv1Rsv3*, *Rsv1Rsv4*, and *Rsv1Rsv3Rsv4* isolines which are resistant to strains G1 to G7. However, the isolines *Rsv3Rsv4* was susceptible to G1 (Maroof et al., 2008).

Given the diversity of *Rsc* genes and *Rsc* locations, and the different types of those genes, functional characterizations are required to understand the molecular bases of *Rsc*-mediated resistance against various SMV SC strains.

Table 3 Summary of the genes that conferring resistance to SMV-SC strains

R gene	**SMV strain**	**Cultivar**	**Location**	**Candidate genes**
Rsc7	SC7	Kefeng No.1	**Chromosome 2** Linked markers (distance): Satt266 (43.7 cM) Satt634 (18.1 cM) Satt558 (26.6 cM) Satt157 (36.4 cM) Satt698 (37.9 cM) Flanking markers: BARCSOYSSR_02_0621 BARCSOYSSR_02_0632	15 candidate genes with one NBS-LRR type gene, one HSP40 gene and one serine carboxypeptidase-type gene
Rsc8	SC8	Kefeng No.1	**Chromosome 2** Flanking markers: BARCSOYSSR_02_0610 BARCSOYSSR_02_0616 Other markers: ZL-42 and ZL-52	*Glyma02g13310*,*Glyma02g13320*, *Glyma02g13400*,*Glyma02g13460*,*Glyma02g13470* *Glyma02g121500* and *Glyma02g121600* (encoding MADS-box proteins)
Rsc5	SC5	Kefeng No1	**Chromosome 2** Flanking markers: Bin 352 Bin353	11 candidate genes with *Glyma02g13495* as the most plausible candidate
Rsc20	SC20	Qihuang-1	**Chromosome 13** Flanking markers: BARCSOYSSR_13_1099 BARCSOYSSR_13_1185	TIR-NBS-LRR type R genes: *Glyma13g194700* and *Glyma13g195100*

Rsc12	SC12	Qihuang-22	**Chromosome 13** Flanking marker: Satt334 Sct_033	
Rsc3	SC3	Qihuang-1	**Chromosome 13**	*Glyma13g25920,Glyma13g25950, Glyma13g25970, and Glyma13g26000*
Rsc3Q	SC3	Qihuang-1	**Chromosome 13** Flanking markers: BARCSOYSSR_13_1114 BARCSOYSSR_13_1136	*Glyma13g25730,Glyma13g25750,Glyma13g25950,Glyma13g25970,* and *Glyma13g26000*
Rsc14Q	SC14	Qihuang-1	**Chromosome 13** Flanking markers: Sat_234 Sct_033 Other markers: Satt334 MY750	
Rsc18	SC18	Kefeng No.1 Qihuang-22	**Chromosome 2** Flanking marker: BARCSOYSSR_02_0667 BARCSOYSSR_02_0670 **Chromosome 13** Flanking marker: SOYHSP176 Satt334	*Glyma02g127800, Glyma02g128200* and *Glyma02g128300*
Rsc4	SC4	Dabaima	**Chromosome 14** Flanking markers: BARCSOYSSR_14_1413 BARCSOYSSR_14_1416	NB-LRR genes*: Glyma14g38510* and *Glyma14g38560* P450 family gene: *Glyma14g38580*

INDEPENDENT HOST FACTORS INVOLVED IN SOYBEAN-SMV INTERACTION

Several independent host factors with defense roles are involved in soybean-SMV interaction (Table 4). The GmEF1A protein is hijacked by SMV P3 protein to promote SMV replication, evidenced by inhibition of SMV accumulation on the *GmEF1A* silenced plants (Luan et al., 2016). Mitogen-activated protein kinase (MPKs) cascades are universal signal transduction that involved in response to various biotic and abiotic stresses, hormone, cell division and developmental processes (Ichimura et al., 2002). GmMPK4, a homolog of mitogen-activated protein kinase-4 in soybean, negatively regulates SA accumulation and defense responses (Liu et al., 2011). Silencing of *GmMPK4* resulted in stunted phenotype, and cell death on the leaves and stems in the silenced plants. In addition, increase of SA and H_2O_2 accumulation were observed in the *GmMPK4* silenced plants (Liu et al., 2011). Silencing of *GmMPK6* in soybean plants via BPMV-mediated VIGS caused stunted growth and spontaneous cell death on the systemic leaves of silenced plants but not on the systemic leaves of empty vector control (Liu et al., 2014). A significant increase of pathogenesis-related (*PR*) genes and the conjugated form of SA were also observed in the silenced plants, suggesting that defense response is

activated in *GmMPK6* silenced plants even before the virus infection (Liu et al., 2014). Challenge inoculation on *GmMPK6* silenced plants using SMV and downy mildew demonstrated that silencing *GmMPK6* enhance resistance against SMV and downy mildew, indicating that *GmMPK6*, similar as *GmMPK4*, is a negative regulator of soybean defense response (Liu et al., 2014). Interestingly, transiently overexpression of *GmMPK6* in *N.benthamiana* or stable overexpression of *GmMPK6* in transgenic *Arabidopsis* results in HR-like cell death even without virus infection (Liu et al., 2014). *GmMPK6* was highly expressed and *PR* genes were highly induced in the transgenic *Arabidopsis* plants, suggesting the positive role of *GmMPK6* in defense response (Liu et al., 2014). These results suggesting complexity function of *GmMPK6* as both repressor and activator in defense responses.

Cytochrome B5 (*GmCYB5*), a gene from a class of heme proteins associated with the endoplasmic reticulum in soybean, reduce SMV-SC15 accumulation (Luan et al., 2019). In response to infection with SMV-SC15, the expression of *GmCYB5* is upregulated to a much greater degree in the resistant cultivar (RN-9) than in the susceptible cultivar (NN1138-2). Silencing of the *GmCYB5* gene promotes SMV-SC15 accumulation in soybean RN-9. GmCYB5 physically interacts with the P3 protein of SMV-SC15 at the cell periphery and is suggested to interfere the role of P3 in SMV replication (Luan et al., 2019).

Apart from individual genes involved in SMV-soybean interaction, the antiviral RNA silencing pathway has been also reported to be involved in soybean resistance to SMV (Alazem et al., 2018a; Alazem et al., 2019; Kontra et al., 2016). The viral replication intermediate, i.e., double-stranded (ds) RNA, is sensed by RNase type III-like enzymes called Dicer-like (DCL) proteins, which cleave the dsRNA into primary short interfering (si) RNAs of 21–24 nucleotides (nt) (Sharma et al., 2013). Viral-derived siRNAs (vsiRNAs) are loaded into the RNA-induced silencing complex (RISC), where they guide argonaute proteins (AGO) to cleave the viral RNA genes upon perfect complementation between vsiRNA and viral genes (Li et al., 2016; Sharma et al., 2013). In the *Rsv3*-cultivar L29, several genes in the antiviral RNA silencing pathway were induced in response to infection by the avirulent strain G5H, but showed no change or even downregulation in response to infection by the virulent strain G7H (Alazem et al., 2018a). This indicates that the antiviral RNA silencing pathway contributes to the ER against G5H. In addition, ABA treatment of soybean or *Arabidopsis* plants induces several genes in the antiviral RNA silencing pathway, which indicates that ABA acts upstream of the RNA silencing pathway and downstream of the Rsv3 sensor protein (Alazem et al., 2019; Alazem et al., 2019). Interestingly, the effect of ABA in

RNA silencing genes was stronger in Rsv3 plants then in rsv-null plants (Alazem et al., 2019)

Micro RNAs (miRNAs) encoded by plant genes target several host, including NB-LRR resistance genes**,**in order to regulate plant responses to different stimuli (Park & Shin, 2015). The tobacco resistance gene N is regulated by *miR6019* and *miR6020*, while the potato PYV resistance gene *Ry* is regulated by *miR482b* (Li et al., 2012). The profiling of miRNAs in the soybean cultivar Williams 82 (*rsv*), which is susceptible to SMV, and in soybean cultivar P196983 (*Rsv1*), which is resistant to SMV-G2 but susceptible to SMV-G7, revealed that *miR168* was upregulated only in the G7-infected PI96983 line and that the upregulation was associated with an LSHR (Chen et al., 2015). *miR168* regulates expression of *AGO1*, a key RNA-slicer enzyme in the antiviral RNA silencing pathway (Mallory et al., 2009). In another example, tomato infected with turnip crinkle virus (TCV), cucumber mosaic virus (CMV), or tobacco rattle virus (TRV) exhibited decreased levels of *miR482*, which allowed the transcript levels of targeted NLRs to increase (Park & Shin, 2015).

Levels of other hormones such as cytokinins and brassinosteroids and expression levels of their related genes were mildly elevated in response to infection by SMV-G5H or SMV-G7H. Cytokinins and brassinosteroids have various functions in plant growth and development, and also increase plant

tolerance to infection by some viruses (Alazem & Lin, 2015; Alazem et al., 2018a; Planas-Riverola et al., 2019).

Several soybean transgenic lines have been developed for SMV resistance (Table 5). These transgenic lines were generated either by overexpressing the resistance genes, or by introducing of SMV genetic elements to induce as pathogen-derived resistance (PDR) (Sarma et al., 2016). Recent study documented a transgenic soybeans that targeting soybean endogenous gene, eIF4E, via RNA interference approach (Gao et al., 2019). The eIF4E has been identified as the major susceptible factor for RNA viruses, including potyviruses (Bastet et al., 2017). Yeast two-hybrid analysis and bimolecular fluorescence complementation assays showed interaction of eIF4E1 with SMV Vpg in the nucleus and with SMV Nia-Pro/NIb in the cytoplasm, suggesting the involvement of eIF4E to promote SMV infection and multiplication (Bastet et al., 2017). Generation of transgenic soybean plants silenced for eIF4E1 showed robust and broad spectrum resistance in T1 and T2 generation against SMV-SC3, SC7, SC15, SC18 and SMV-R (Bastet et al., 2017). Hence, further discoveries of the R genes and host factors involved in SMV-resistance can bring new insights on the development of cultivars with a broader spectrum resistance to SMV.

Table 4 Summary of host factor/gene involve in resistance to SMV

Host factors	**Roles in SMV resistance**	**Reference**
eEF1A	Targeted by P3, promotes SMV replication	(Luan et al., 2016)
GmEDR1, *GmEDS1* *GmPAD4*	Induce accumulation of SA, mediated resistance against SMV	(Zhang et al., 2012)
GmHSP90	Reduced the replication and movement of SMV-G2 (*Rsv1*-mediated resistance)	(Zhang et al., 2012)
*WRKY*6 *WRKY30*	*Rsv1*-mediated resistance against SMV-G2	(Zhang et al., 2012)
GmPP2C3a	Induces callose accumulation, restrict SMV movement	(Seo et al., 2014)
GmPEX14	Induces burst of H_2O_2, (*Rsc15*-mediated resistance)	(Rui et al., 2017b)
GmMPK4	Negatively regulate the SA accumulation and defense response	(Liu et al., 2011)
GmMPK6	Repressor and activator in defense response	(Liu et al., 2014)
GmKR3	Stimulating ABA accumulation	(Xun et al., 2019)
GmCYB5	Target the P3 protein to inhibit SMV accumulation	(Luan et al., 2019)

Table 5 Summary of SMV-tolerant cultivar

Tolerance cultivar		**Reference**
Transgenic *GmAKT2*	Alter the level of potassium, reduce the spread of SMV	(Zhou et al., 2014)
RNAi-mediated silencing of SMV P3 transgenic soybean	Exhibited stable and enhanced resistance to SMV SC3 and other potyviruses.	(Yang et al., 2018)
Transgenic GmKR3	Enhances resistance against multiple viruses, including SMV-SC3, via ABA signaling	(Xun et al., 2019)
Attenuated SMV-Coat-protein mediated-resistance transgenic soybean	Highly resistant to SMV strain D and A (in Japan)	(Furutani et al., 2006)
SMV-CP-RNAi transgenic soybean	Induces a functional gene silencing system and resulted in a viral-resistant phenotype.	(Kim et al., 2013)
Inverted repeat-SMV-*HC-pro* transgenic soybean	Induced RNA-mediated resistance via RNAi by targeting SMV-*HC-pro*	(Gao et al., 2015)
Soybean RNA interfere lines, silenced for eIF4E	Interferes viral replication cycles, increase broad-spectrum resistance against SMV-SC3, SC7,SC-15,SC18, and SMV-R	(Gao et al., 2019)

CONCLUSIONS AND FUTURE PERSPECTIVES

Several studies have been carried out to characterize SMV-soybean interactions leading to the identification of several *R*-genes such as the *Rsv* and *Rsc* genes as well as few other individual genes required for resistance (Hajimorad & Hill, 2001; Liang et al., 2010; Luan et al., 2019; Maroof et al., 2010; Redekar et al., 2016a; Zhan et al., 2006). However, the molecular mechanisms underlying many of which are still lacking, and further investigations would help understand how resistant cultivars achieve resistance against various SMV strains so it can be transferred to susceptible cultivars or species (Luan et al., 2019; Zhou et al., 2014). Nonetheless, many new SMV strains have also emerged with counter-defense weapons evolved over natural selection in the field (Choi et al., 2005b; Koo et al., 2005b). Their abilities to break high-specific resistance also require further investigations to determine elements involved in breaking resistance, which in many cases involved recognition avoidance by R-protein (Choi et al., 2005b; Koo et al., 2005b). A good breeding-for-resistance strategy would aim to develop cultivars with resistance against wide range of strains, where new molecular tools, such as CRISPR/Cas9 (which knocks out specific genes by deletion) or RNAi (which silences specific genes) can speed up the breeding program. The use of

CRISPR/Cas9 in generating lines disrupted with eIF4E proved successful in generating with cucumber plants resistance to zucchini yellow mosaic virus and papaya ring spot mosaic virus-W (Chandrasekaran et al., 2016). In addition, the use or RNAi technique to generate transgenic lines expressing fragments from SMV genes has also been successful (Table 5). For instance, transgenic soybean lines expressing part of the P3 and HC-pro genes showed a stable and enhanced resistance to SMV-SC3, -SC7, -SC15, -SC18, and -R (a novel recombinant strain found in China), and have the potential to significantly increase soybean yield (Kim et al., 2016; Yang et al., 2018). With the continuous discoveries of defense mechanisms, and the implementation of new molecular tools in breeding programs generating efficient resistant plants will sounds faster to achieve.

In this study, I disclosure two host factors that works antagonistically during SMV infection. The first host factor which was identified in *G. max*, named as GmPAP2.1, demonstrated strong resistance to SMV strain G5H and delays infection of SMV strain G7H. I characterized this host factor and described thoroughly in the second chapter. The third chapter of this study describes the HSP70 family protein members that are essential for SMV infection in *N. benthamiana* and probably also essential for SMV infection in *G. max* following the observation of increase transcription level of the genes upon

SMV infection. Identifying the host factors that associate in the viral infection; either the one that hinder or promote infections, and understanding on how these host factors interact with SMV proteins may provide new insight on the management of SMV infection.

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Chapter 2

Overexpression of a purple acid phosphatase GmPAP2.1 confers soybean mosaic virus resistant in a susceptible soybean cultivar

[2] This chapter is a slightly modified version of paper that has been accepted in *Journal of Experimental Botany,* erab496, https://doi.org/10.1093/jxb/erab496

ABSTRACT

A purple acid phosphatase (PAP) from soybean cultivar L29, GmPAP2.1, may function as a resistance factor against specific strains of soybean mosaic virus (SMV). Overexpression of GmPAP2.1 from the SMV-resistant cultivar L29 conferred viral resistance to a susceptible cultivar Lee 74. GmPAP2.1 interacts with the SMV protein P1 in the chloroplast, resulting in the upregulation of the *ICS1* gene, which in turn promotes the pathogen-induced salicylic acid (SA) pathway. SA accumulation was elevated in response to the co-expression of GmPAP2.1 and SMV, while transient knock-down of endogenous SA-related genes resulted in systemic infection by SMV-G5H, suggesting that GmPAP2.1-derived resistance depends on the SA-pathway for the activation of a defense response. My findings suggest that the PAP of soybean cultivar L29 functions as an SA-pathway-dependent resistance factor against SMV.

Keywords: SMV, *Glycine max*, purple acid phosphatase, virus resistance, salicylic acid, virus–host interaction, chloroplast

INTRODUCTION

Soybeans originated in China and are now grown in many regions around the world (Qiu & Chang, 2010), and the demand for soybeans is strong and increasing (Wilson, 2008). In addition to being consumed by humans, soybeans are used as livestock and aqua-culture feeds and have multiple industrial uses (Hartman et al., 2011; Liu, 2008; Qiu & Chang, 2010). Unfortunately, soybean production has substantial constraints, one of which is infection by pathogens including plant viruses (Hartman et al., 2011). From 2010 to 2014, the estimated average loss of soybean production across US states and Ontario due to soybean diseases was about $60.66 USD per acre per year (Allen et al., 2017). Among the pathogens that reduce global soybean production, soybean mosaic virus (SMV) is especially difficult to control and causes significant yield losses every year (Hill & Whitham, 2014). SMV, which is a member of the genus *Potyvirus* in the family *Potyviridae*, has a single-stranded positive-sense RNA genome of approximately 9.6 kb (Hajimorad et al., 2018).

SMV was previously classified into seven strains (G1–G7) based on disease reactions on different soybean cultivars (Cho & Goodman, 1979). Following the latter report, researchers have continuously detected new strains

of SMV, including new strains in Southern China (Li et al., 2010) and Poland (Jezewska et al., 2015) as well as G5H (SMV-G5H) and G7H (SMV-G7H) strains, which caused severe disease outbreaks in Korea (Choi et al., 2005; Kim et al., 2003; Kim et al., 2004). Because chemical compounds that can effectively control SMV are lacking, SMV management mostly relies on the use of resistant cultivars. Intensive study of resistance genes in soybean revealed three independent loci, *Rsv1*, *Rsv3*, and *Rsv4*, that confer resistance against SMV strains G1–G7 (Saghai Maroof et al., 2008; Shakiba et al., 2012). Several others resistance loci, including *Rsc4*, *Rsc5*, *Rsc7*, *Rsc8*, *Rsc15*, and *Rsc20*, that confer resistance against SMV-SC strains in China have also been characterized (Karthikeyan et al., 2017; Karthikeyan et al., 2018; Rui et al., 2017; Wang et al., 2011; Wang et al., 2011; Wang et al., 2017).

Soybean cultivar L29 carries a dominant resistant gene, *Rsv3*, on chromosome 14 (Chowda-Reddy et al., 2011; Jeong et al., 2002b). *Rsv3* confers resistance to several strains of SMV including G5, G6, G7, and G5H, but L29 exhibits mosaic symptoms in response to infection by SMV strains G1 to G4 (Choi et al., 2005; Gunduz et al., 2001; Redekar et al., 2016). Resistance-breaking strains of SMV have repeatedly emerged and caused declines in soybean production (Choi et al., 2005; Koo et al., 2005). SMV-G7H, for example, emerged in the late 1990s in Korea; G7H can overcome *Rsv3*-

mediated resistance and causes systemic mosaic symptoms in L29, resulting in substantial loses to South Korea's soybean production (Kim et al., 2003). Considering that RNA viruses like SMV have a high frequency of mutation in their sequences, there is a substantial probability that mutant viruses will not be recognized by resistance (*R*) genes and will be able to overcome *R*-gene-mediated resistance. Research on the SMV proteins responsible for the breaking of *R*-gene-mediated resistance demonstrated that a change of a single amino acid (aa) in the HC-Pro protein of the virus can alter symptom expression in soybean cultivars carrying *Rsv1* or *Rsv3* (Seo et al., 2011). Given that even a single mutation can enable an SMV strain to overcome the resistance of *R* genes, characterization of new resistance genes in soybean plants, especially genes that confer resistance to multiple strains of SMV, is required to secure and improve global soybean production.

In this study, I found that co-expression of the purple acid phosphatase gene (*GmPAP2.1*) from soybean cultivar L29 and an SMV clone in soybean susceptible cultivar Lee 74 inhibits the systemic movement of SMV-G5H and delays infection by SMV-G7H. Furthermore, GmPAP2.1 was found to interact with SMV-P1 protein in the chloroplast, and its expression was induced the transcription of endogenous SA-related genes. Co-expression of GmPAP2.1 and SMV promotes elevation of SA accumulation in co-expression plants, while

transient knock-down of the endogenous salicylic acid (SA)-related genes, *GmICS1* and *GmNPR1*, compromises GmPAP2.1-derived resistance to SMV-G5H. Thus, my results indicate that a GmPAP2.1 from soybean cultivar L29 functions as a resistance factor by interacting with P1 protein of SMV; the interaction upregulates the SA pathway, which in turn generates a resistance response.

MATERIALS AND METHODS

I. Plants growth and virus strains

The *Rsv3*-containing cultivar L29 (*Rsv3*) and the *Rsv3*-null cultivar Lee 74 (*rsv3*), were provided by the National Agrobiodiversity Center, Republic of Korea. Soybean plants were grown in a growth chamber at 25 ^{o}C under a 16/8 h (light/dark) photoperiod. Two SMV strains that used are SMV-G5H and SMV-G7H.

II. Construction of expression clones

To amplify the target gene, *GmPAP2.1*, total RNAs were extracted from leaves of soybean cultivar L29 (*Rsv3*) by the RNAiSO plus reagent method (TaKaRa). Reverse transcription of 2 µg of total RNAs was carried out using GoScript ^{TM} Reverse Transcriptase (Promega) followed by conventional polymerase chain reaction (PCR) amplification reaction to amplify full-length open reading frame (ORF) of GmPAP2.1 using gene-specific primer pair that contained the *Mlu*I restriction enzyme site (5'-GCACGCGTatggatgaaaagaccacta-3' and 5'- GCACGCGTccgaaataatgcaagaga-3'). Sequences of the *GmPAP2.1* specific primers were designed based on the

William 82 soybean reference genome with annotation *Glyma06g028100* from the Soybase (USDA-ARS Soybean Genetics and Genomic Database) (Grant et al., 2009). The PCR product was then digested with *Mlu*I and inserted into the pSMV-G5H::GFP vector, which expresses green fluorescent protein (GFP) as the virus replicates. The resulting clone was named pSMV-G5H::GFP::GmPAP2.1. Subsequently, the expression clone of GmPAP2.1 from cultivar William 82 (*rsv3*) that does not confers resistance to SMV-G5H was constructed in similar way, with the insert that was amplified using total RNAs of soybean cultivar William 82. The resulting clone was named as pSMV-G5H::GFP::GmPAP2.1 (W82). In addition, a soybean housekeeping gene *GmACT11* (*Glyma18g290800*) (Du et al., 2018), was used as a control to test possible effect(s) of an additional ORF on the infectivity of the viral vector. pSMV-G5H::GFP::GmACT11 which contains an additional *GmACT11* ORF amplified from total RNAs of soybean cultivar L29 was constructed similarly.

III. In vitro transcription and translation

In vitro transcription reactions were performed with the T7 RNA polymerase (New England Biolabs). Transcribed RNAs were purified by phenol-chloroform extraction followed by ethanol precipitation. Quality of transcribed RNAs was assessed by NanoPhotometer (Implen) and by gel

electrophoresis. *In vitro* translation was carried out using wheat germ extract (WGE) translation system (Promega) with the presence of FluroroTect^{TM} Green$_{Lys}$ (Promega) for fluorescence labelling and detection, according to manufacturer's instructions. Translated samples were then load on 12% SDS-PAGE gel and subjected for electrophoresis. The fluorescent signal was detected by Typhoon FLA 9500 biomolecular imager (GE Healthcare) using 437 nm laser (excitation 502 nm; emission 530 nm) with LPB filter.

IV. Plant inoculation and visual assessment

To evaluate the effect of the cloned genes on SMV replication and movement, the DNA plasmid of the empty vector pPZP (mock), pSMV-G5H::GFP (infectious clone of SMV-G5H expressing GFP), the pSMV-G5H::GFP::GmPAP2.1 (L29) (expression clone of GmPAP2.1 from soybean cultivar L29), the pSMV-G5H::GFP::GmPAP2.1 (W82) (expression clone of GmPAP2.1 from soybean cultivar William 82), and the pSMV-G5H::GFP::GmACT11 (expression clone of GmACT11 from soybean cultivar L29) were inoculated into fully expanded unifoliate leaves of 2-week-old plants of soybean cultivar Lee 74 (*rsv3*; with 10 μg of total DNA plasmids per leaf and two leaves per plant). Symptom development was visually assessed at 10, 14, and 21 dpi in the observation of GmPAP2.1 resistance against SMV-G5H

and -G7H, and only at 14 dpi for other visual assessments of symptom development. Experiments were performed with three biological replicates with at least three plants for each replicate.

V. Quantitative RT PCR (RT-qPCR)

Total RNAs were extracted from the upper non-inoculated leaves by the RNAiSO plus reagent method (TaKaRa, Japan). Equal amounts of total RNAs (2 µg) were used for cDNA synthesis using GoScript ™ Reverse Transcriptase (Promega) and Oligo(dT)$_{15}$ primer. Technical triplicate RT-qPCR reactions were prepared using IQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instruction in a CFX384 Real-Time PCR detection system (Bio-Rad). Each reaction mix (10 µl) consisted of 20 ng of cDNA, 5 µl of 2 X IQ™ SYBR Green Supermix, and 10 pmoles of each primers. The qPCR was performed as follows: 95 ^{o}C for 3 min, followed by 40 cycles at 95 ^{o}C for 10 s, 59 ^{o}C for 30 s and melting curve data was obtained by increasing the temperature from 55 ^{o}C to 95 ^{o}C for 1 s/step. Two endogenous reference genes were used as reference genes to normalize qPCR results. *GmELF1B* and *CYP2* were used to normalize qPCR results of *GmACT11* overexpression samples, and *Actin* and *CYP2* were used to normalize qPCR results of other sample sets. Melt curve analysis was carried out using the Bio-

Rad CFX manager V1.6.541.1028 software (Bio-Rad). Each sample was evaluated in at least three independent experiments, including three technical replicates. The primer sets used for qPCR are show in Table. 2.

VI. Sequence analysis of GmPAP2.1 homologs

Specific primer pairs (Table. 1) were used to amplify the *GmPAP2.1* gene sequences from the following soybean cultivars: William 82, L29, Somyungkong, V94-5152, Harosoy, Geumjeongkong-2, and Lee 74. The PCR products were cloned into pGEM® -T Easy Vector Systems (Promega) and transformed into TOP10 Chemically Competent *E. coli* cells. Purified plasmid DNA was sequenced by Macrogen Inc. Sequence alignment and phylogenetic analysis were performed using ClustalW and the neighbor-joining and bootstrap methods implemented in MEGA X (Kumar et al., 2018).

VII. Protein domain search and phylogenetic analysis of GmPAP2.1

To identify the protein domain of GmPAP2.1, I determined the protein sequence of GmPAP2.1 and compared it with the domains in the Conserved Domain Database of the NCBI (Marchler-Bauer et al., 2014). Protein structure was predicted by the I-TASSER server (Yang & Zhang, 2015). To determine the

homology of GmPAP2.1 among proteins in vascular plants, a BLASTP search of the GmPAP2.1 protein was performed against the vascular plant protein database. The sequence of hits and their percentage of similarity were used to construct a phylogenetic tree using neighbor-joining and bootstrap methods in MEGA X (Kumar et al., 2018).

VIII. Subcellular localization analysis in epidermal cells of *N. benthamiana*

The transient expression vector pBin61 (Bendahmane et al., 2002) with eGFP or mCherry tagging was used to express GmPAP2.1 and SMV P1 in *N. benthamiana*. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and agro-infiltrated into the leaves of *N. benthamiana* as described previously (Bendahmane et al., 1999) with minor modification. At 2 dpi, fluorescence microscopy (emission at 509 nm and 610nm, exposure 400 ms) was used to assess GFP and mCherry expression in the epidermal cell. Co-localization between proteins were evaluated by performing Pearson's correlation coefficient (PCC), Mander's overlap coefficient (MOC), and cytofluogram in ImageJ software (Rueden et al., 2017).

IX. Nuclear localization assay

For relocalization assay, NLS sequence (PKKKRKV) of SV40 was incorporated into the C-terminal end of GmPAP2.1. A specific primer pair was used to amplify the insert (Table. 1). The PCR product was then digested with *Xba*I and *Bam*HI and inserted into pBIN61 with mCherry tag for visualization of the nuclear localization purpose. The construct was transformed into *Agrobacterium* competent cell and agro-infiltrated into *N. benthamiana*. At 2 dpi, the mCherry fluorescence signal was observed under fluorescence microscope (Leica). For challenge inoculation purpose, the PCR product was digested with *Mlu*I, inserted into pSMV-G5H::GFP viral vector generating pSMV-G5H::GFP::GmPAP2.1-NLS, and transformed into TOP10 Chemically Competent *E. coli* cells. A 10 µg quantity of purified plasmid was used to inoculate the primary leaves of the soybean cultivar Lee 74.

X. Genes knock-down constructs

Two endogenous SA-related genes, *GmICS1* (*Glyma01g104100*) and *GmNPR1* (*Glyma15g127200*), one ABA-related gene, *GmABA2* (*Glyma11g151400*), and the *GmPDS* gene (*Glyma18g003900*) were partially amplified (300 bp) from the soybean cultivar Lee 74 and *GmPAP2.1*

(*Glyma06g028100*) was partially amplified (300 bp) from soybean cultivar L29. The products were digested using *BamHI* and *Sal*I restriction enzymes and then inserted into the open reading frame of the RNA 2-encoded polyprotein of bean pod mottle virus (BPMV) (Zhang et al., 2009).

XI. Inoculation of the knock-down constructs and challenge inoculation of pSMV-G5H::GFP::GmPAP2.1 or pSMV-G5H::GFP

A 5 μg quantity of the recombinant BPMV RNA 2 plasmid (pBPMV) containing the gene of interest (SA- and ABA-related genes, and the *GmPDS* gene) and 5 μg of BPMV RNA 1 were used to inoculated the primary leaves of the soybean cultivar Lee 74. Sample from each knock-down construct was collected at 13 dpi following observation of the PDS knock-down symptoms. Following confirmation of the knock-down level of each construct, a 10 μg quantity of pSMV-G5H::GFP::GmPAP2.1 was used to inoculated the upper non-inoculated leaves of SA- and ABA- related genes transiently knocking-down Lee 74, and a 10 μg quantity of pSMV-G5H::GFP was used to inoculated the *GmPAP2.1* transiently knocking-down L29.

XII. Yeast two-hybrid and beta-galactosidase assays

For yeast two-hybrid (Y2H) assays, *GmPAP2.1* and its mutants were cloned into pACT2 (AD), and SMV-G5H proteins were cloned into pAS2-1 (BD). The construct with the combination of AD and BD was then co-transformed into yeast strain AH109, which was grown on plates lacking leucine and tryptophan (SD-Leu/-Trp) for 2 days at 30 ^{o}C. Single colonies were selected and grown on SD-Trp/Leu broth medium into 0.5 at OD_{600} and transferred on SD-Leu/-Trp, or SD-His/-Leu/-Trp, or SD-His/-Leu/-Trp/-Ade agar medium in serial dilution 10^{0}, 10^{-1}, and 10^{-2} for 2 days at 30 ^{o}C. For the β-galactosidase assay, a filter assay with X-gal as the substrate for β-galactosidase was used (Paiano et al., 2019). The filter membranes were incubated at 30 ^{o}C for 2 days until a blue color appeared.

XIII. Construction of expression clone for transient expression in *N. benthamiana*

SMV-P1, *GmPAP2.1*, and *GmRieske Fe/S* (accession number: AM498291.1) genes were amplified using specific primer pairs (Table. 2). PCR-amplified fragments were digested with *Mlu*I restriction enzyme, purified using Nucleospin PCR clean up and gel extraction (Macherey-Nagel), and inserted into pPZP binary vector. The positive clones were further transformed

into GV3101 *Agrobacterium* Electro-competent Cells (GoldBio).

XIV. Co-immunoprecipitation (Co-IP) assay

For Co-IP assay, SMV-P1, GmPAP2.1 and GmRieske Fe/S proteins were transiently co-expressed by agroinfiltration in *N. benthamiana* plants as previously described (Muñoz & Castellano, 2018), albeit some minor modifications. At 2 dpi, 0.2 g of leaf samples were collected and extracted in ice-cold protein extraction buffer (250 mM NaCl, 100 mM $MgCl_2$, 5 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol, 25 mM Tris-Cl, pH 7.5, 0.3% Triton-X 100, 1 mM PMSF, and 1 x protease inhibitor cocktail (Roche)). Leaf lysates were then load into HisTrap HP His tag protein purification columns (Cytiva, US), washed 10 column volumes using binding buffer (20 Mm NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4), and eluted with elution buffer (20 Mm NaH_2PO_4, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The eluents were subjected for SDS-PAGE in a 12% polyacrylamide gel and detected using anti-His antibody, anti-HA antibody (cell signaling technology, USA), and anti-FLAG antibody (Sigma) at a dilution of 1: 10.000 (v/v).

XV. Measurement of SA level

SA was extracted from 0.3 g of the 24 hpi soybean leaf tissues and measured by reverse-phase HPLC on a Prominence HPLC system (Shimadzu, Japan) with a C18 column, as previously described (Verberne et al., 2002), albeit some modifications. In short, the tissues were ground into powder with the presence of liquid nitrogen and homogenized two times by 1 ml and 500 µl of 90% and 100% methanol respectively. After centrifugation, a 5 µl of 0.4 M NaOH was added into the supernatant and homogenized. The methanol was evaporated in SpeedVac concentrator for 1 h, and the remaining residues were resuspended in 500 µl of acetate buffer (pH 5.6). A 5% final concentration of Trichloroacetic acid (Sigma-aldrich, US) was added into 250 µl volume of resuspension and homogenized. A 1 ml of ethyl acetate:cyclopentane with 1% (v/v) isopropanol was used to partitioned the resuspension. The organic phases were evaporated in SpeedVac concentrator and the remaining residues were dissolved in 500 µl of 100% methanol and were filtered before subjected to HPLC analysis. The diode-array detection was applied (SA was detected at wavelength 280 nm, retention time 20 min). A 2,3-diaminopropionic acid was used as an internal standard. The mobile phase was acetate buffer (pH 3.6)/ methanol (72:28, v/v) with a flow rate of 1 ml/min. Three independent biological replicates were performed for each data point.

XVI. Statistical analysis

Experiments were conducted at least three times (replicates) with three individual plants for each replicate. Means of treatments were compared using ANOVA with Tukey's HSD post Hoc Test in SPSS. The graphs were generated using GraphPad Prism (USA). The differences were considered significant at $p < 0.05$.

Table 1 List of primers used for amplification of inserts.

No	Primer name	Forward (5'→3')	Reverse (5'→3')	Purpose
1	*GmPAP2.1*	GCACGCGTatggatgaaaagaccacta	GCACGCGTccgaaataatgcaagaga	Co-expression with SMV
2	*GmPAP2.1* (△1-256aa)	GCACGCGTatgcatagtccaatgtataatagttatgtga	GCACGCGTccgaaataatgcaagagat	Co-expression with SMV
3	*GmPAP2.1* (△1-125aa)	GCACGCGTcctcctcctgttggcc	GCACGCGTccgaaataatgcaagagat	Co-expression with SMV
4	*GmPAP2.1* (△36-125aa)	1.GCACGCGTatggatgaaaagaccacta	1.aaatacaacatcgactttattattccaca	Co-expression with SMV
		2.gctccagtttacataacgattgg	2.GCACGCGTccgaaataatgcaagagat	
		3.GCACGCGTatggatgaaaagaccacta	3.GCACGCGTccgaaataatgcaagagat	
5	*GmPAP2.1* (A305V)	1. GCACGCGTatggatgaaaagaccacta	1. tttgatAcccgtttagatcgttcat	Co-expression with SMV
		2. aacgggTatcaaatattgcata	2.GCACGCGTccgaaataatgcaagagat	
		3. GCACGCGTatggatgaaaagaccacta	3.GCACGCGTccgaaataatgcaagagat	
6	*GmPAP2.1* (H295A & H297A)	1. GCACGCGTatggatgaaaagaccacta	1. tcgttcataagcgGCaacgGCaccagcaaataca	Co-expression with SMV

		2. gtatttgctggtGCcgttGCcgcttatgaacgatctaaac	2. GCACGCGTccgaaataatgcaagagat	
		3. GCACGCGTatggatgaaaagaccacta	3. GCACGCGTccgaaataatgcaagaga	
7	*GmPAP2.1* (I337A)	1. GCACGCGTatggatgaaaagaccacta	1. tccttctGCatttcctccatctcc	Co-expression with SMV
		2. ggaaatGCagaaggactggctact	2. GCACGCGTccgaaataatgcaagagat	
		3. GCACGCGTatggatgaaaagaccacta	3. GCACGCGTccgaaataatgcaagagat	
8	*NtrbcL*	CGGGATCCatgtcaccacaaacaga	CGGTCGACcttatccaaaacgtc	Subcellular localization in *N. benthamiana*
9	*GmICS1*	CGGGATCCaagcactagattgccaaa	GCGTCGACatacttctgtttctgcaatt	Knockdown
10	*GmNPR1*	CGGGATCCcagagcaggggaaag	GCGTCGACtttcattgagatcaacctcc	Knockdown
11	*GmABA2*	CGGGATCCggtggcttaggacc	GCGTCGACtccacttatatatttagcatcatc	Knockdown
12	*GmPDS*	CGGGATCCgaattccttctacgtactgcc	CCCTCGAGgggagagaaatggttcct	Knockdown
13	P1	GCTCTAGAatggcaacaatcatgatt	CGGGATCCgtagtgctgaatatcatcc	Subcellular localization in *N. benthamiana*

14	*GmPAP2.1*-NLS	GCTCTAGAatggatgaaaagaccactac	CGGGATCCtacctttctcttctttttggccgaaataatgcaagaga	Nuclear localization assay/ subcellular localization
15	*GmPAP2.1*-NLS	GCACGCGTatggatgaaaagaccacta	GCACGCGTtacctttctcttctttttggccgaaataatgcaagaga	Co-expression with SMV
16	*GmACT11*	GCACGCGTatggcagacgctga	GCACGCGTgaagcactttctgtgc	Co-expression with SMV
17	Flag-*GmRieske Fe/S*	GCACGCGTgactacaaagacgatgacgacaagatggcatccaccact	GCACGCGTagcccaccatggagc	Transient expression in *N. benthamiana* for co-IP
18	HA-*GmPAP2.1*	GCACGCGTtacccatacgatgttccagattacgctatggatgaaaagacc	GCACGCGTccgaaataatgcaagaga	Transient expression in *N. benthamiana* for co-IP
19	6xHis-P1	GCACGCGTcaccaccaccaccaccacatggcaacaatcatga	gcacgcgtgtagtgctgaatatcatccat	Transient expression in *N. benthamiana* for co-IP
20	T7 P1-Protease	taatacgactcactatagggatggcaacaatcat	attctccacattaggaggca	*In vitro* transcription

Sequences are indicated by lowercase letters, while additions of restriction enzyme sequences are indicated by uppercase letters. Nucleotide changes for substitution mutants are indicated by red uppercase letters. The additional of nuclear localization signal sequence are indicated by underline

Table 2 List primers used for RT-qPCR.

No	Primer name	Forward (5'→3') [a]	Reverse (5' → 3')	Reference ID
1	*GmPAD4*	atgacccttcctcaatc	gtatgaaactatgtcttcatcac	*Glyma13g069800*
2	*GmNPR1*	atggcttattcagccg	aaggttaggtccaggg	*Glyma15g127200*
3	*GmICS1*	atggcaatgggcac	atggtattggaggaagtatat	*Glyma01g104100*
4	*GmEDS1*	Cggacttcttcggg	gagtgaaggaaattggg	*Glyma04g177700*
5	*GmZEP1*	atggctactaccttatgttac	gccaacaacaaaaggt	*Glyma17g174500*
6	*GmZEP2*	atggctcctaccttga	atgactgacacaaggtg	*Glyma11g055700*
7	*GmABA2*	atgtccactactggtact	gatatggaagaggcgc	*Glyma11g151400*
8	*GmABA2b*	atgtctactaccggtaatg	gatatggaagagacgcac	*Glyma11g151700*
9	*GmPAP2.1*	atgcctattttagttggaat	agatgtttgttctgggtgggt	*Glyma06g028100*
10	SMV-CP	aaggctgcagctctctcggg	tcacatcccttgcagtatgcctt	FJ807701.1
11	*GmACT11*	atcttgactgagcgtggttattcc	gctggtcctggctgtctcc	*Glyma18g290800*
12	*GmCYP2*	cccctccactacaaaggctcg	Cgggaccagtgtgcttcttca	*Glyma12g024700*
13.	*GmELF1B*	tgggaaaacctatatttctggg	Tttggcaggtgcagcttca	*Glyma02g276600*

[a] Primers were designed with a length of about 100 bp based on the sequence of each gene according to the reference ID.

RESULTS

I. Characterization of GmPAP2.1

During early infection of the SMV-resistant soybean cultivar L29 (carrying *Rsv3*) by SMV-G5H, upregulation of several genes based on the RNA sequencing was observed in the current study and was previously reported (Accession number: Bioproject PRJNA723692) (Alazem et al., 2018; Seo et al., 2014). Among the upregulated genes, a gene with reference ID *Glyma06g028100* exhibited relatively constant upregulation during early infection by SMV strain G5H (8 to 54 h post-infection, hpi) (Fig. 1). For validation of the RNA-Seq data (Seo et al., 2014), real-time quantitative (qPCR) was conducted using a *Glyma06g028100*-specific primer pair. As expected, the expression level of *Glyma06g028100* was steadily upregulated during early infection of soybean cultivar L29 by G5H but was only temporarily upregulated during early infection of soybean cultivar L29 by G7H (Fig. 2A). Interestingly, the expression level of *Glyma06g028100* in the *rsv3*-null soybean cultivar Lee 74 was not significantly different from that in the healthy control (Fig. 2B). These results confirm that *Glyma06g028100* is upregulated only in soybean cultivar L29 upon SMV-G5H infection.

In this study, I obtained a sequence of *Glyma06g028100* that was assembled from soybean cultivar William 82 from the soybean database (https://www.soybase.org/). *Glyma06g028100* putatively encodes a protein that has the highest similarity to the purple acid phosphatase 2 (PAP2) of *Glycine soja* (Fig. 3A). *Glyma06g028100* was therefore named *GlymaPAP2.1*. According to the gene map, *GmPAP2.1* is located at chromosome 6, between markers sat_130 and BARC_024137_04780 (Fig. 3B). Previously, in total 35 *PAP* genes were identified from soybean (*G. max*) genomes (Li et al., 2012). Phylogenetic analysis showed that among these PAPs, *GmPAP2.1* is most closely related to *Glyma06g03100.1* (Fig. 4). Analysis on the presence of the conserved domain indicated that both of GmPAP2.1 from soybean cultivar L29 and William 82 contain an FN3 domain and a metaphosphate C domain in its N- and C-terminal regions, respectively (Fig. 5).

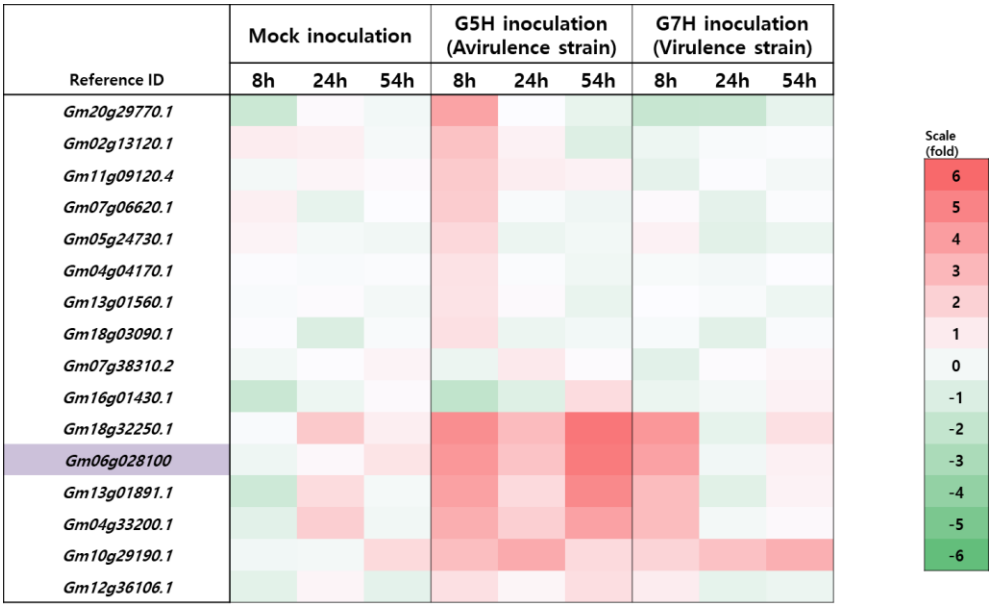


Fig. 1. Upregulation of several genes in soybean cultivar L29 due to the SMV infections. Heat map depicting the upregulation of genes during the early infection (8, 24, and 54 hour post inoculation) of SMV strain G5H and G7H (Accession number: Bioproject PRJNA723692) (Alazem et al., 2018; Seo et al., 2014). *Gm06g028100* (*GmPAP2.1*) was steadily upregulated during the infection of G5H and temporarily upregulated during G7H infection.

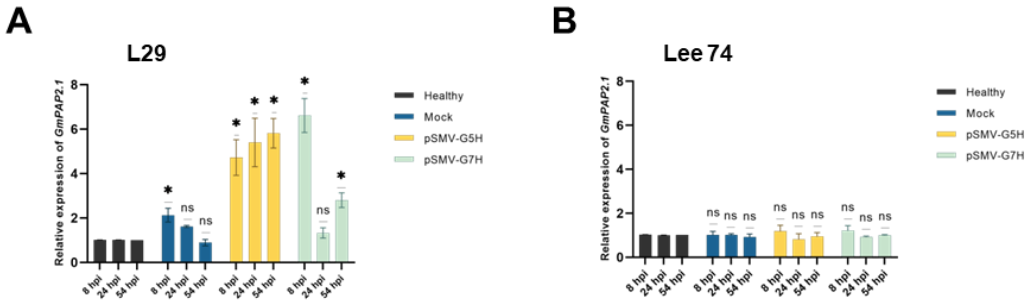


Fig. 2. Expression levels of GmPAP2.1. Expression of *GmPAP2.1* in soybean cultivar L29 (A) and Lee 74 (B) following inoculation with the SMV strains G5H or G7H. In (A) *GmPAP2.*1 was significantly and steadily increased by inoculation with G5H but fluctuated by inoculation with G7H. In (B), *GmPAP2.1* was not significantly affected by inoculation with G5H or G7H. Values in a and b are means ± SD from three independent experiments. An asterisk indicates a significant differences and ns indicates non-significant difference between SMV-inoculated plants and healthy control ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

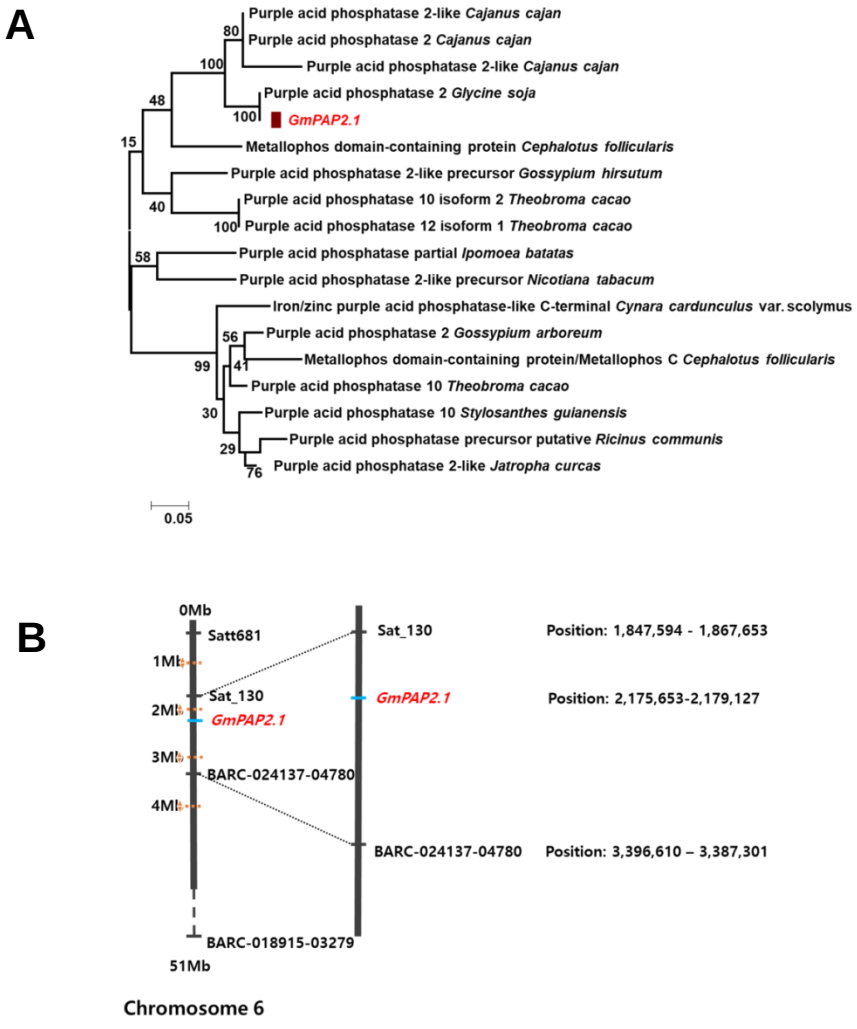


Fig. 3. Phylogenetic relationships of GmPAP2.1 proteins among higher plants and its approximate position on the gene map. (A) phylogenetic tree indicating that the protein encoded by *GmPAP2.1* in soybean cultivar L29 is closely related to the purple acid phosphatase 2 of *Glycine soja*. (B) Approximate position of *GmPAP2.1* on the gene map in between two markers i.e. Sat_130 and BARC-024137-04780.

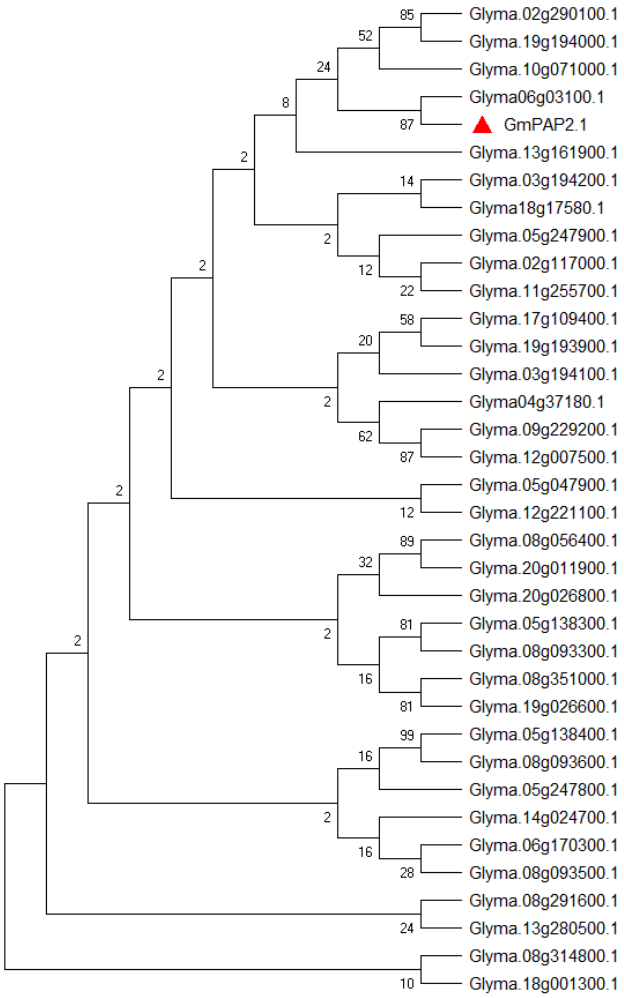


Fig. 4. A phylogenetic tree of PAPs in soybean (*Glycine max*). Sequences of the soybean PAPs proteins were obtained from Soybase (USDA-ARS) Soybean Genetics and Genomic Database (Grant et al., 2009). Phylogenetic analysis was conducted in MEGA X (Kumar et al., 2018). According to the analysis, *GmPAP2.1* (*Glyma06g028100*) is mostly related to *Glyma06g03100.1*.

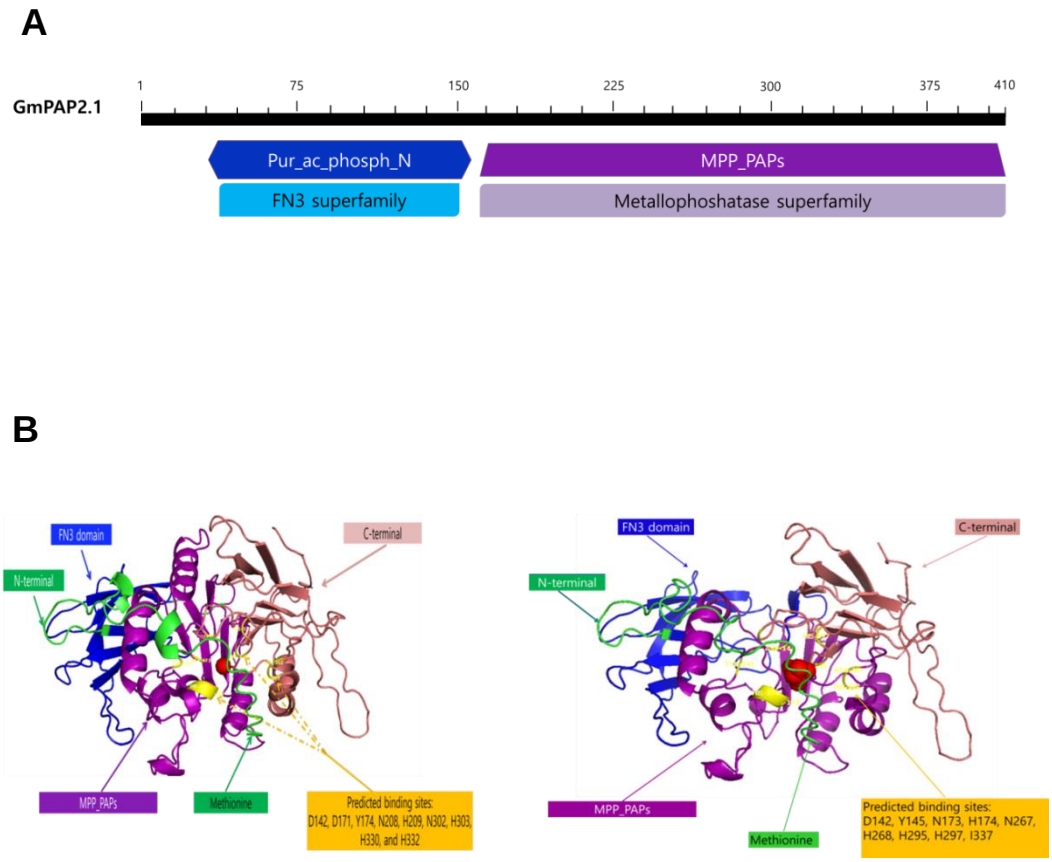


Fig. 5. Prediction of conserved domain and protein structure of GmPAP2.1. In (A), the protein encoded by *GmPAP2.1* belongs to the purple acid phosphatases of the metallophosphatase superfamily. It consists of purple acid phosphatase on the N terminal and metallophosphate on the C terminal. In (B), prediction protein structure of GmPAP2.1 from William 82 (left) and from L29 (right). Green indicates the location of the start codon; blue indicates the location of PAP on the N terminal; and pink indicates the C terminal. The predicted binding sites are indicated by yellow.

II. Analyzes of nucleotide sequence and the predicted structure of GmPAP2.1 from cultivar L29 and other cultivars

I analyzed the sequence of *GmPAP2.1* from several cultivars (L29, William 82, Lee 74, Somyungkong, V94-5154, Geumjeongkong-2, and Harosoy) and identified differences in nucleotide sequences of L29 and those of the other cultivars. The *GmPAP2.1* coding sequence (CDS) is 105 bp shorter in L29 than in the other cultivars. Nucleotides 421 to 525 of the *GmPAP2.1* coding sequence are present in the other cultivars but are missing in L29 (Fig. 6A). This deletion reduced the number of aa residues of GmPAP2.1 from 445 residues in the other cultivars to 410 residues in L29. Alignment of the deduced aa of GmPAP2.1s from soybean cultivars identified an aa substitution at residue 305 into alanine (A) in the GmPAP2.1 of L29 from valine (V) in the other cultivars (Fig. 6B). The deletion of 35 amino acid residues in the N-terminal region of GmPAP2.1 in L29 altered the predicted structure of the GmPAP2.1 protein at the C-terminal region by having an additional predicted binding site, a region where the other molecule(s) might bind, at position 337 which is absent in the other cultivars (Fig. 7).

Identification of binding sites is an onset for the function identification of protein (Kinoshita & Nakamura, 2005). Using structure-based programs, consensus approach (COACH) (Yang et al., 2013), which recognize ligand

binding sites from the BioLiP-a protein function database (Yang et al., 2012), I identified the predicted ligand binding sites of GmPAP2.1 from cultivar L29 at aa residues D142, Y145, N173, H174, N267, H268, H295, H297, and I337, and at aa residues D142, Y174, N208, H209, N302, H303, H330, and H332 in GmPAP2.1 from cultivar William 82 (Fig. 5B). Among the identified binding sites, the presence of binding site at aa residue I337 in the C-terminal region of GmPAP2.1 from L29 makes it distinct in compare to those from the other cultivars including William 82 (Fig. 7A, red circle). Changes of the predicted protein structure in the C-terminal region of GmPAP2.1 from L29 might provide the distinctive ability to recognize and bind with SMV protein, which results in the differences of protein function of GmPAP2.1 from L29 than those from the other soybean cultivars.

In addition, phylogenetic analysis using the deduced aa sequences of the *GmPAP2.1*s showed that the GmPAP2.1 of L29 formed a distinct branch relative to the GmPAP2.1 of the other cultivars (Fig. 8). Together these results suggest that the GmPAP2.1 of L29 is different from that of the other cultivars in this study and that this difference might be related to the resistance conferred by GmPAP2.1.

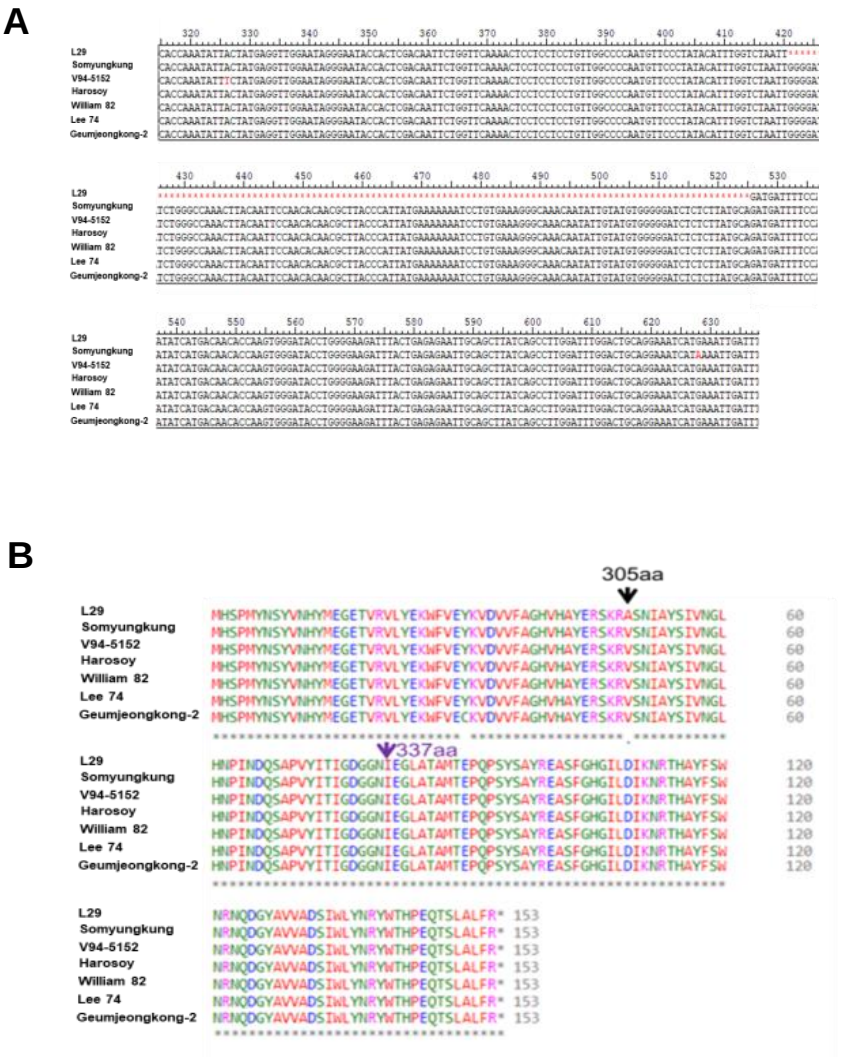


Fig. 6. Analyses of nucleotide sequences and corresponding amino acid sequences of *GmPAP2.1* from L29 and six other cultivars (Somyungkong, William 82, Harosoy, Lee 74, V94-5152, and Geumjeongkong-2). In (A), nucleotide sequences of the N terminals of *GmPAP2.1*. The L29 sequence is missing 105 bp of coding region's nucleotides at 421-525 that are present in the other cultivars (indicated by red asteriks). (B) Alignment of amino acid (aa) sequences of C terminal regions of GmPAP2.1. The black arrow indicates an aa in L29 that differs from that in the other cultivars; the purple arrow indicates the position of the aa in L29 that is predicted to be a binding site that bind into viral protein (P1).

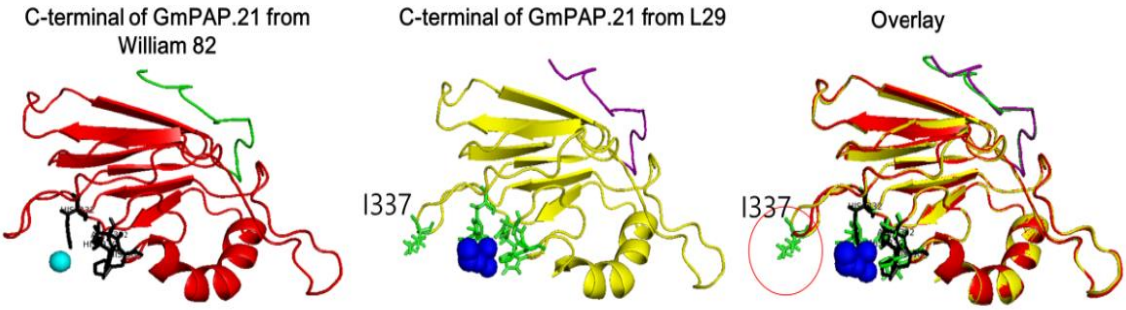


Fig. 7. Prediction protein structure of C-terminal of GmPAP2.1. The binding site (I337) that might bind into viral protein only present in the GmPAP2.1 of L29 (red circle) but not in the William 82.

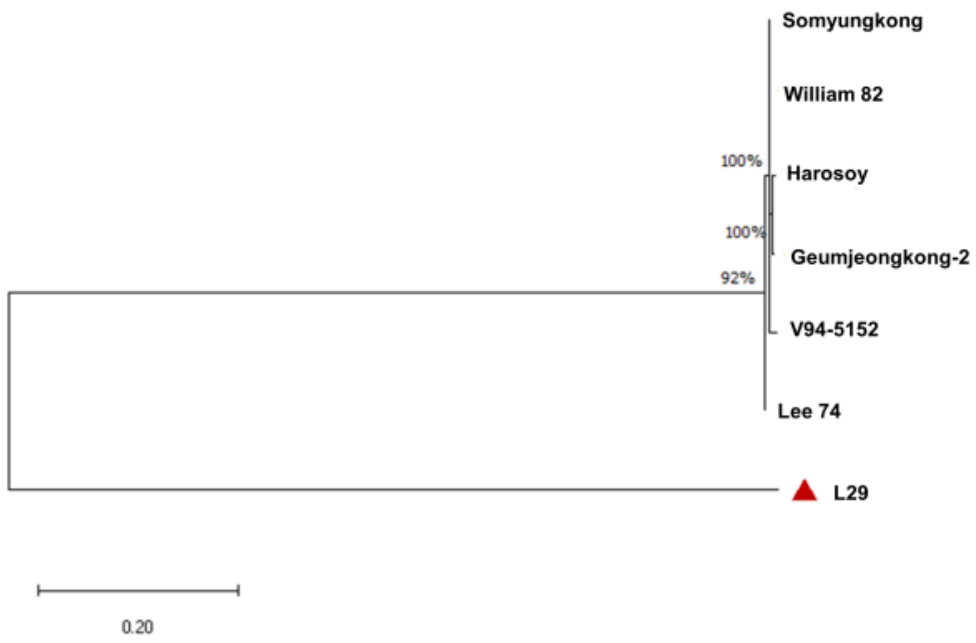


Fig. 8. Phylogenetic tree of *GmPAP2.1* sequences of the seven cultivars. The L29 sequence is located on a different branch than the sequences of the other cultivars.

III. Overexpression of GmPAP2.1 from L29 conferred viral resistance to a susceptible cultivar Lee 74

To investigate the potential effect of the GmPAP2.1 on SMV infectivity, I inoculated the primary leaves of the susceptible soybean cultivar Lee 74 with the DNA plasmids of the SMV infectious cDNA clones (Seo et al., 2009) expressing green fluorescent protein (GFP; pSMV-G5H::GFP); and expressing G5H, GmPAP2.1, and GFP (pSMV-G5H::GFP::GmPAP2.1) (Fig. 9A, 9B). Two and three weeks after inoculation, viral infection was assessed based on the expression of GFP (Fig. 10A). Soybean plants co-expressing the GmPAP2.1 protein and SMV-G5H or SMV-G7H did not show any symptoms but virus-only inoculated plants (pSMV-G5H::GFP and pSMV-G7H::GFP) showed a typical rugose mosaic symptom (Fig. 9C).

As indicated by observation of the inoculated leaves with normal UV light and with a fluorescence microscope, the intensity of GFP expression was decreased in the plants that co-expressed GmPAP2.1 (pSMV-G5H::GFP::GmPAP2.1; Fig. 9D). RT-qPCR of total RNAs extracted from the inoculated leaves revealed that viral RNA accumulation was significantly lower (nearly 10-fold lower) in the GmPAP2.1 co-expression plants (pSMV-G5H::GFP::GmPAP2.1) than in the pSMV-G5H::GFP inoculated plants (Fig. 10C). Neither the expression of GFP nor the replication of viral RNA was detected in the upper non-inoculated leaves (Fig. 10A, 10D) of the GmPAP2.1

co-expression plants.

To determine whether GmPAP2.1 contributes to suppressing G7H, a more virulent strain of SMV, I inoculated Lee 74 with G7H rather than with G5H. Both GFP expression and viral RNA accumulation were significantly decreased in the upper non-inoculated leaves of GmPAP2.1 co-expression plants at 21 dpi than in the pSMV-G7H::GFP inoculated plants (Fig. 10B, 10E). Together these results indicated that overexpression of GmPAP2.1 significantly inhibited virus replication in the inoculated leaves and may also inhibit systemic movement of the SMV-G5H and inhibited or delayed infection by G7H.

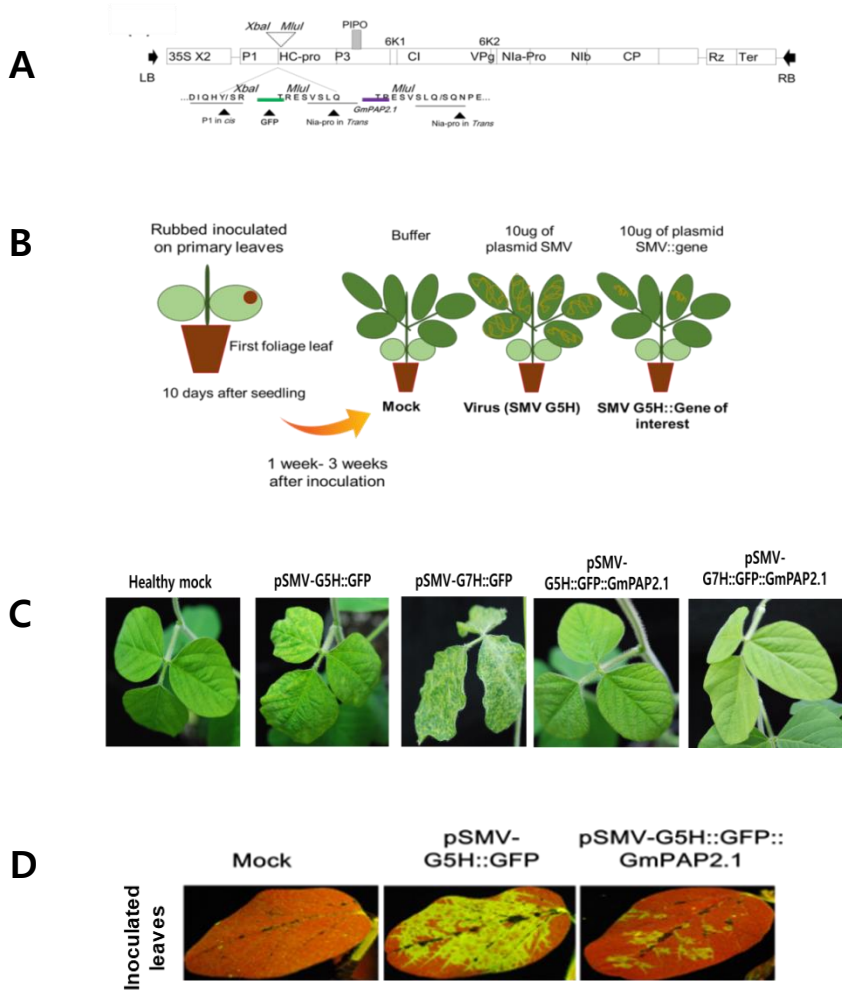


Fig. 9. Schematic representation of pG5H::GFP::GmPAP2.1 construction and of its inoculation into soybean. (A) GmPAP2.1 was inserted by using the *Mlu*I restriction enzyme site on the MCS next to the location of the GFP reporter gene. (B) A 10-µg quantity of plasmid was rub-inoculated on the first emerged primary leaves using carborundum. (C) Phenotype of soybean cultivar Lee 74 inoculated with pSMV-G5H::GFP, pSMV-G7H::GFP, pSMV-G5H::GFP::GmPAP2.1 and pSMV-G7H::GFP::GmPAP2.1 at 14 dpi. Typical mosaic symptoms were observed on the upper non-inoculated leaves of plants inoculated with wild type viral vector (pSMV-G5H::GFP and pSMV-G7H::GFP) but not on mock control plants or on the co-inoculation plants. (D) Observation of inoculated leaves of soybean cultivar Lee 74 that were inoculated with only the vector (pSMV-G5H.GFP) or with pSMV-G5H::GFP::GmPAP2.1 at 14 dpi. GFP expression was detected in both vector only inoculated plants and GmPAP2.1 co-expression plants.

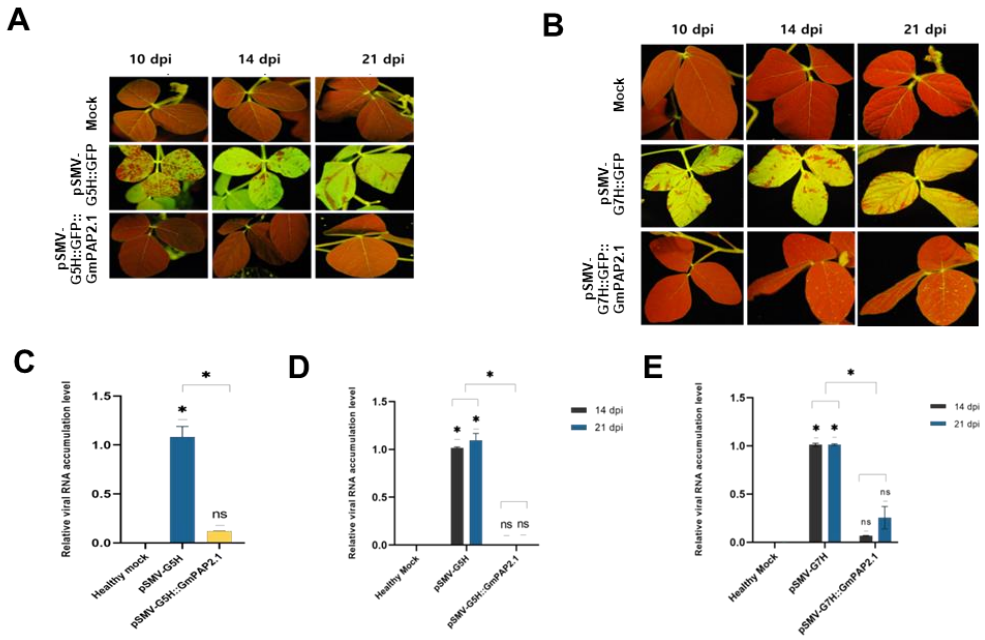


Fig. 10. Symptoms and virus accumulation in Lee 74 plants inoculated with only the vector (pSMV-G5H::GFP or pSMV-G7H::GFP) and with GmPAP2.1 co-expressing vector (pSMV-G5H::GFP::GmPAP2.1 or pSMV-G7H::GFP::GmPAP2.1). (A) Observation on upper non-inoculated leaves. GFP expression (indicated by green) was detected in the vector-only inoculated plants (pSMV-G5H.GFP) but not in the GmPAP2.1 co-expression plants. (B) Upper non-inoculated leaves of Lee 74 plants. GFP expression was detected in both vector-only (pSMV-G7H::GFP) inoculated plants and in the GmPAP2.1 co-expression plants. (C) Viral RNA accumulations in the inoculated leaves at 14 dpi. (D), (E) Quantification of viral RNA accumulation in upper non-inoculated leaves at 14 and 21 dpi, respectively. Values in (C, D, and E) are means $\pm$ SD from three independent experiments. Asterisk indicates a significant difference between vector-only inoculated plants and mock plants or GmPAP2.1 co-expression plants. "ns" indicates non-significant differences relative to the mock plants ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

IV. Addition of GmPAP2.1 right downstream of the SMV-P1 protein does not affect P1 protease activity

It has been shown that the potyviral P1 and its protease domain positively regulate potyvirus infections (Ivanov et al., 2014; Pasin et al., 2014). To test possible effect(s) of an additional ORF right downstream of P1 on protease activity of SMV P1, *in vitro* cleavage analyses has been conducted. *In vitro* translation assay demonstrated that an addition of GmPAP2.1 ORF downstream of SMV P1 in the viral vector does neither alter nor reduce P1 protease activity. P1 protein maintained its protease activity without or with the presence of GmPAP2.1 (Fig. 11). These results indicate decrease or absent of SMV viral loads in the upper non-inoculated leaves of Lee 74 inoculated with SMV vector co-expressing GmPAP2.1 is not caused by defective P1 protease activity in the viral vector that reduce infectivity of viral vector but due to the presence of GmPAP2.1 inhibiting or preventing SMV replication.

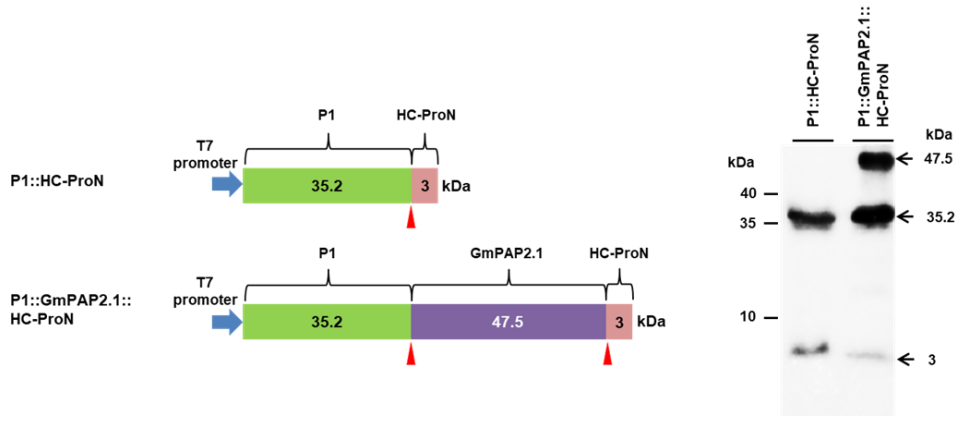


Fig. 11. *In vitro* cleavage analyses of SMV P1. (A) Schematic representation of plasmids used for *in vitro* transcription. Red arrow heads indicate cleavage sites of SMV P1. (B) *In vitro* translation to evaluate protease activity of P1. *In* vitro translation was performed using wheat germ extract. Fluorescence-labelled translation products were separated by SDS-PAGE and detected using fluorescence imager. Protein bands with their respective size (right) are evidence of P1 self-cleavage activity indicating that an addition of GmPAP2.1 open reading frame in the viral vector (P1::GmPAP2.1::HC-ProN) did not affect protease activity of P1 protein. Molecular weight markers are shown in left side of the gel image.

V. GmPAP2.1 interacts with the SMV-P1 protein in the chloroplast

To determine the interaction between GmPAP2.1 and SMV proteins, I conducted yeast two-hybrid assays and found that GmPAP2.1 specifically interacts with the P1 protein of SMV (Fig. 12A). A ß-galactosidase assay was then conducted to confirm the interaction between GmPAP2.1 and the P1 protein; a change in color was evident for the yeast that incorporated GmPAP2.1 and P1 (Fig. 12B). Further, I conducted co-IP assay to validate the *in vivo* interaction between GmPAP2.1 and SMV-P1 protein. As shown in Fig. 4C left panel, GmPAP2.1 only precipitated with the presence of SMV-P1 protein demonstrating that GmPAP2.1 interacted with SMV-P1 *in vivo*. Previous report showed the interaction between SMV-P P1 and the host *Pinellia ternate's* Rieske Fe/S protein of the cytochrome *b6/f* complex and suggested possible role of this interaction in symptom development (Shi et al., 2007). To determine whether there is competitions between Rieske Fe/S protein and GmPAP2.1 to bind to P1, I conducted Co-IP in the absence or presence of GmRieske Fe/S. Analysis of relative band intensity of Co-IP samples indicated that the interaction between GmPAP2.1 and P1 protein does not seem to be affected by the presence of GmRieske Fe/S while showing stronger interaction than the interaction between P1 protein and GmRieske Fe/S (Fig. 12C).

I also conducted subcellular localization assay to determine the

localization of GmPAP2.1 and its co-localization with P1 protein. At 2 dpi, fluorescence microscopy indicated that GmPAP2.1 is chloroplast-localized protein and that it is co-localized with P1 in the chloroplast (Fig. 12D). Pearson's correlation coefficient (PCC) and Mander's overlap coefficient (MOC) of GmPAP2.1 and chloroplast marker showed values of 0.982 and 0.999, respectively, while GmPAP2.1 and P1 showed value of 0.944 and 0.981, respectively. The PCC and MOC values that obtained from the co-localization assay showed a high correlation between proteins confirming that GmPAP2.1 and P1 protein co-localized in the chloroplast. Subsequently, I also observed the localization of GmPAP2.1 from cultivar William 82, however, unlike GmPAP2.1 from L29, it does not co-localized with P1 protein in the chloroplast (Fig. 13A).

To evaluate the importance of chloroplast localization in the GmPAP2.1-derived resistance, I tried to re-localize GmPAP2.1 into nucleus by incorporating the simian virus 40 (SV40) T antigen nuclear localization signal (NLS) peptide sequence (PKKKRKV) (Cao et al., 2017) that was fused into C-terminal region of GmPAP2.1. Observation of the *N. benthamiana* leaves tissues infiltrated with the mCherry::GmPAP2.1-NLS protein using fluorescence microscope showed, as expected, localization of many mCherry::GmPAP2.1-NLS proteins in the nucleus with PCC and MOC values

up to 0.788 and 0.715, respectively (Fig. 14A). I also observed that the mCherry::GmPAP2.1-NLS proteins were not completely transported into nucleus as some of proteins were still expressed in the chloroplast (Fig. 13B).

To validate that transportation of GmPAP2.1 into nucleus might disrupt the GmPAP2.1-derived resistance, I co-expressed GFP::GmPAP2.1-NLS with SMV-G5H (pSMV-G5H::GFP::GmPAP2.1-NLS) on Lee 74. At 14 dpi following co-expression, the GFP signals were observed in the upper-non inoculated leaves of the wild type pSMV-G5H::GFP inoculated plants. Interestingly, the GFP signals were also observed in pSMV-G5H::GFP::GmPAP2.1-NLS inoculated plants. No GFP expression was observed in the mock or pSMV-G5H::GmPAP2.1 inoculated plants (Fig. 14B). Quantification of the viral RNA accumulation also demonstrated that detectable level of SMV viral RNA was accumulated in the pSMV-G5H::GFP::GmPAP2.1-NLS inoculated plants whereas none in the mock nor in pSMV-G5H::GFP::GmPAP2.1 inoculated plants (Fig. 14C). Altogether these results suggest the importance of the chloroplast co-localization of GmPAP2.1 and P1 protein in the GmPAP2.1-derived resistance.

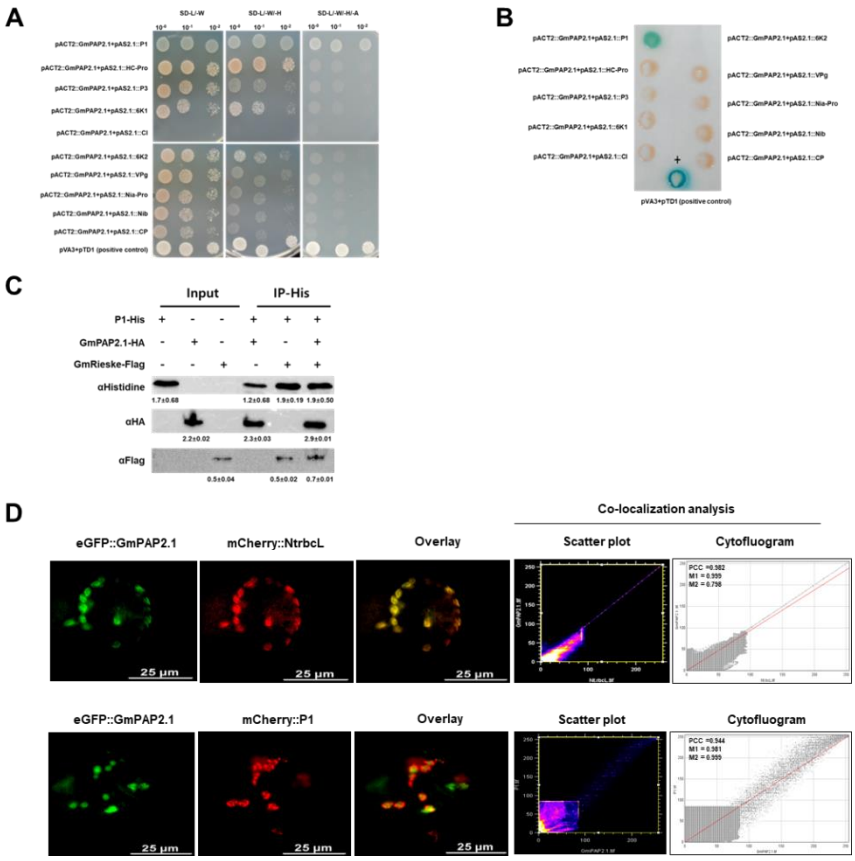


Fig. 12. Interactions between GmPAP2.1 and SMV proteins. In (A), only yeast cells expressing the combination of GmPAP2.1 and P1 could grow on the tryptophan, leucine, histidine, and adenine drop-out medium, indicating a strong interaction between the two proteins. In (B), among the 10 proteins of SMV, only P1 interacted with GmPAP2.1, as indicated by the blue color of the yeast cells. In (C), P1 with His tag (P1-His), GmPAP2.1 with HA tag (GmPAP2.1-HA), and GmRieske Fe/S with Flag tag (GmRieske-Flag) that were expressed in *N. benthamiana* leaves were analyzed by co-immunoprecipitation (Co-IP). GmPAP2.1-HA and GmRieske-Flag were only precipitated in the presence of P1-His. Value under each band is band density relative to the rubisco as a loading control. In (D), the leaves were examined at 2 days post co-infiltration, and fluorescence (indicating expression of GFP for GmPAP2.1 and mCherry for P1 and chloroplast marker) was assessed by confocal microscopy. Scale bar=25µm. Co-localization analyses were performed by using ImageJ software. The values in (C) are means ± SD from three independent measurement of proteins bands.

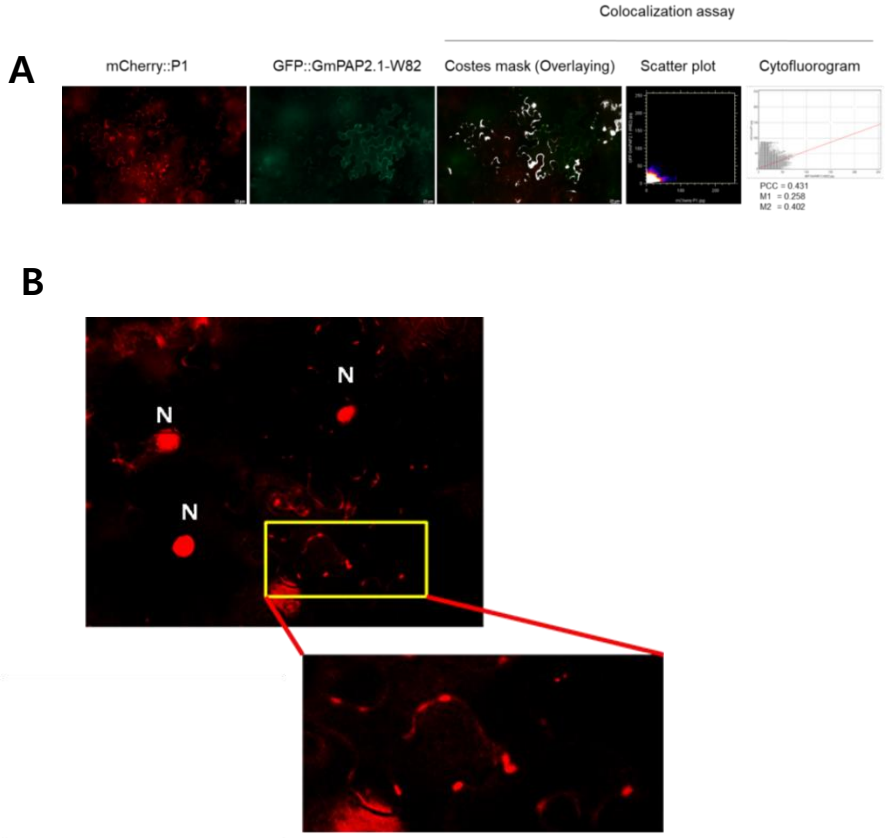


Fig. 13. Localization of GmPAP2.1 from cultivar William 82 and GmPAP2.1 with NLS tag. (A) Localization of GmPAP2.1 from soybean cultivar William 82 in *N. benthamiana*. Co-localization analyses were performed by using ImageJ software. PCC (Person's correlation coefficient) value is low 0.431, suggesting GmPAP2.1 from William 82 does not co-localized with P1 in the chloroplast. (B) Some proteins of GmPAP2.1 are still localized in the chloroplast (an enlarged image).

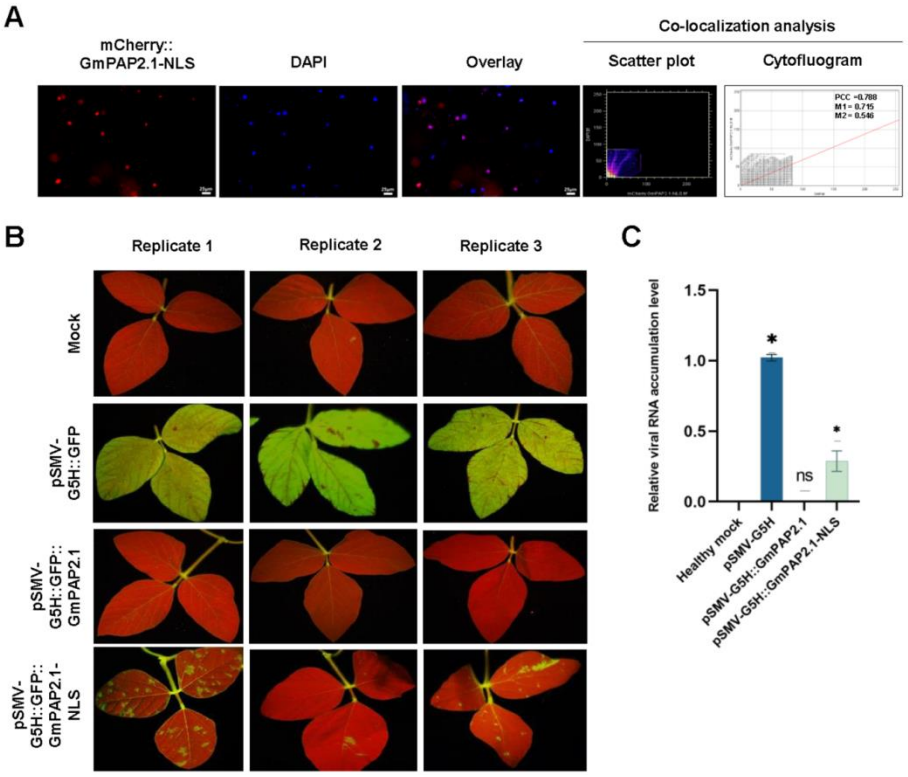


Fig. 14. Nuclear localization of GmPAP2.1. In (A), fluorescence images of GmPAP2.1 from leaf mesophyll cells of *N. benthamiana* plants at 2 dpi. DAPI stains the nucleus (blue), red signals are from GmPAP2.1-NLS-mCherry. Scale bar=25μm. Co-localization analyses were performed by using ImageJ software. PCC value is relatively high (0.788) suggesting high degree co-localization between nucleus and GmPAP2.1-NLS. In (B), the nuclear localized GmPAP2.1 (*GmPAP2.1-NLS*) was co-expressed with SMV-G5H in soybean cultivar Lee 74. The GFP expression represents viral accumulation, and (C) quantification of the viral accumulation in upper non-inoculated leaves at 14 dpi. SMV-G5H accumulation was detected in the upper non-inoculated leaves of the *GmPAP2.1-NLS* overexpression plants, unlike in the *GmPAP2.1* overexpression plants. Values are means ± SD from three independent experiments. An asterisk indicates a significant difference between mock and vector-only inoculated plants. "ns" indicates non-significant differences of *GmPAP2.1* and *GmPAP2.1-NLS* overexpression plants relative to the mock plants ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

VI. The C-terminal region of GmPAP2.1 is crucial for resistance against SMV infection

To identify the region of the GmPAP2.1 from L29 that is responsible for resistance against SMV, I constructed several deletion and site-directed mutants (Fig. 15) and co-expressed them in soybean cultivar Lee 74 after cloning each mutant into pSMV-G5H::GFP. As shown in Figure 16, mutants with N-terminal deletions (△1–125aa and △1–256aa), an internal deletion (△36–125aa), and a double-alanine substitution (H295A & H297A) did not disrupt the resistance induced by GmPAP2.1. Observation of the GFP expression demonstrated that virus replication and spread caused by pSMV-G5H::GFP::GmPAP2.1 were similar with or without these mutations in the inoculated leaves and in the upper non-inoculated leaves. However, when I deleted the C-terminal region of GmPAP2.1 (△257–324 aa), co-expression of the resulting mutant resulted in increased GFP expressions in both the inoculated leaves and in the upper non-inoculated leaves, indicating the disruption of resistance to SMV-G5H. This disruption was stronger with the complete deletion (△257–409 aa) than with the partial deletion (△257–324 aa) of the C-terminal region of GmPAP2.1.

Co-expression of an aa-substitution mutant of the predicted binding site (I337A) of L29 GmPAP2.1 that absent in other cultivars (Fig. 7) with SMV-

G5H, resulted in increased GFP expression in the upper non-inoculated leaves, which was similar to the result obtained with the complete deletion of the C-terminal of GmPAP2.1 in L29 (Fig. 16A). A single aa-substitution mutant at 305 aa (A305V) resulted in a moderate amount of systemic movement of the virus to the upper non-inoculated leaves, which was similar to the result obtained from partial deletion in C terminal region (△257–324 aa) of GmPAP2.1 (Fig. 16A). Consistently, RT-qPCR result demonstrated an accumulation of SMV-G5H in the upper non-inoculated leaves of partial deletion of the C-terminal region (△257-324 aa), aa-substitution mutant at 305 aa (A305V) and at 337 (I337A), and complete deletion of the C-terminal region (△257–409 aa) inoculated plants at 14 dpi (Fig. 16B).

In addition, yeast two hybrid analysis showed that the interaction between P1 and mutant GmPAP2.1 only occurs when the C-terminal present with the isoleucine (I) residue at position 337; evident by yeast colonies appears white and grow on SD-His/-Leu/-Trp/-Ade agar medium (Fig. 16C). Due to the yeast strain, AH109, used in this experiment that carries a mutation inhibiting the adenine synthesis pathway, when the interaction is absent, colonies appear pink to reddish-brown (Paiano et al., 2019), and white when the interaction is occurs. Together, these results suggest that the C-terminal region of the L29 GmPAP2.1 is crucial for interaction with SMV-P1 protein and in the resistance response to SMV infection.

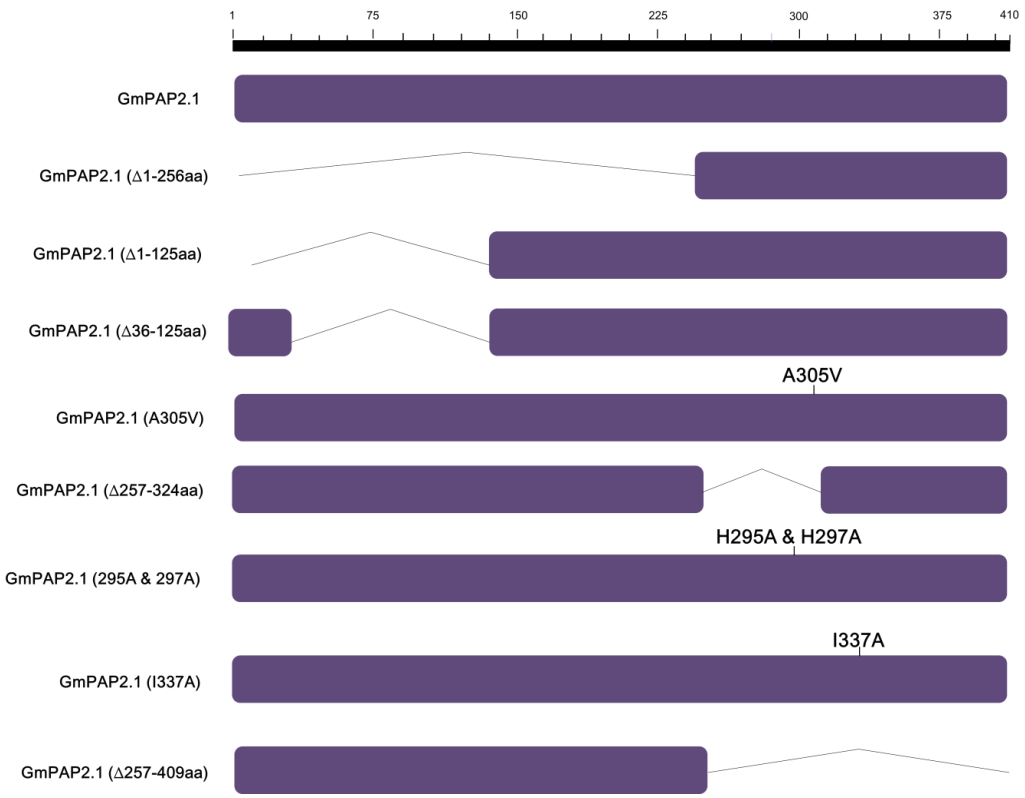


Fig. 15. Schematic diagram of mutant construction in GmPAP2.1. The deletions and amino acid substitutions are indicated.

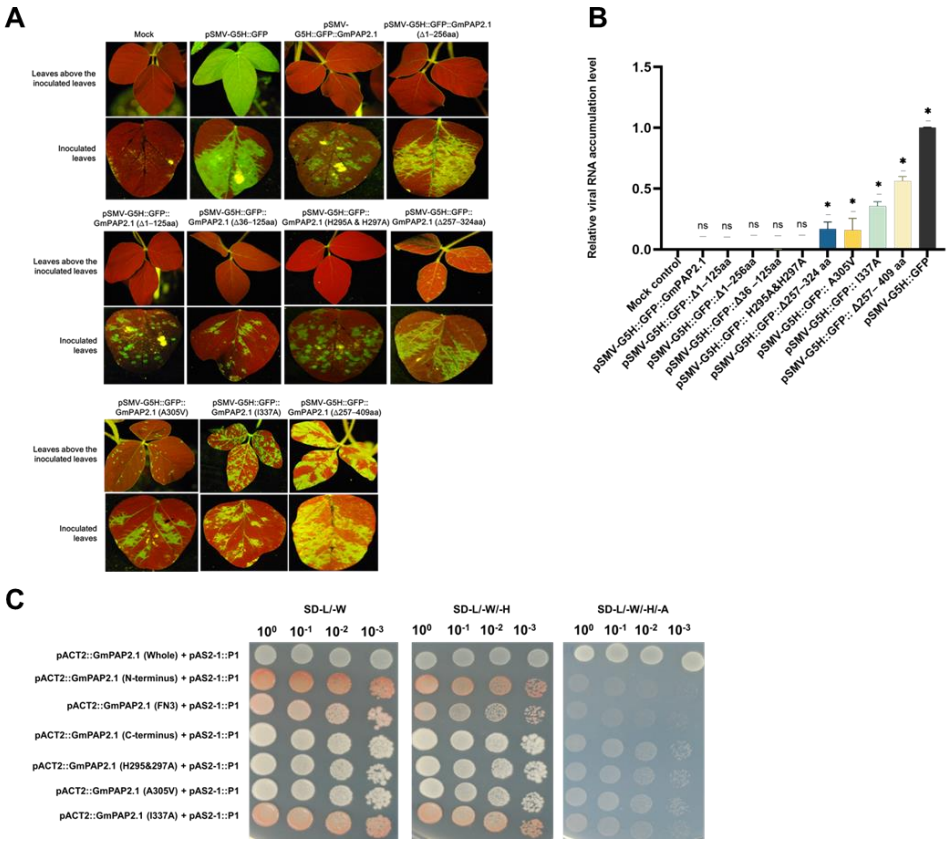


Fig. 16. Effects of co-expression of *GmPAP2.1* mutants with SMV-G5H in soybean cultivar Lee 74. In (A), GFP expression in inoculated leaves and in leaves above the inoculated leaves were observed with UV light. Among the mutants, mutants with deletion of 257–324 aa and 257–409 aa, and amino acid substitution mutants at aa residues 305 and 337 in the C terminal of GmPAP2.1 had a significantly reduced ability to cause resistance, as indicated by the expression of GFP in the leaves above the inoculated leaves. In (B), quantification of viral accumulation level in upper non-inoculated leaves of *GmPAP2.1*'s mutants co-expression plants at 14 dpi. In (C), the interaction between SMV-P1 protein and *GmPAP2.1* mutants were observed by yeast two hybrids. Values in (B) are means ± SD of three independent experiments. Asterisks indicate differences relative to the mock plants, whereas "ns" indicates non-significant differences relative to the mock plants ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

VII. GmPAP2.1s from another cultivar than L29 does not confer resistance against SMV

Given that GmPAP2.1 from L29 confers resistance against SMV through its interaction with SMV-P1 protein, I evaluated the interaction of SMV-P1 and GmPAP2.1s from other soybean cultivars (William 82, SMK, Lee 74, Harosoy, Hardee, Geumjeongkong-2, and V94-5152) by yeast two hybrid analysis. Among all yeast colonies, only the one that incorporated GmPAP2.1 from L29 and P1 grow on SD-His/-Leu/-Trp/-Ade agar medium, indicates only GmPAP2.1 from L29 is strongly interacts with P1 (Fig. 17A). To validate that only GmPAP2.1 from soybean cultivar L29 provides resistance against SMV, I constructed the expression clone pSMV-G5H::GFP::GmPAP2.1 (W82), in which the GmPAP2.1 was amplified from soybean cultivar William 82. To determine effect(s) of an additional ORF in the viral vector on infectivity, I also constructed expression clone pSMV-G5H::GFP::GmACT11 that co-expressing non-resistant host gene which has similar size as GmPAP2.1 and used as control. Co-expression of the clones in Lee 74 result in higher expression level of *GmPAP2.1 (W82)* and *GmACT11* genes in co-expression plants than in the mock control, which indicated that both genes were indeed upregulated on the co-expressed plants (Fig. 17B). Unlike co-expression of the GmPAP2.1 of L29, however, co-expression of the GmPAP2.1 of W82 or GmACT11 did not affect SMV infection, i.e., both GFP expression and viral accumulation were observed

in the upper non-inoculated leaves (Fig. 17C, 17D). These results suggest that only GmPAP2.1 from L29 interacts with SMV-P1 and that this interaction is important in the GmPAP2.1-derived resistance to SMV.

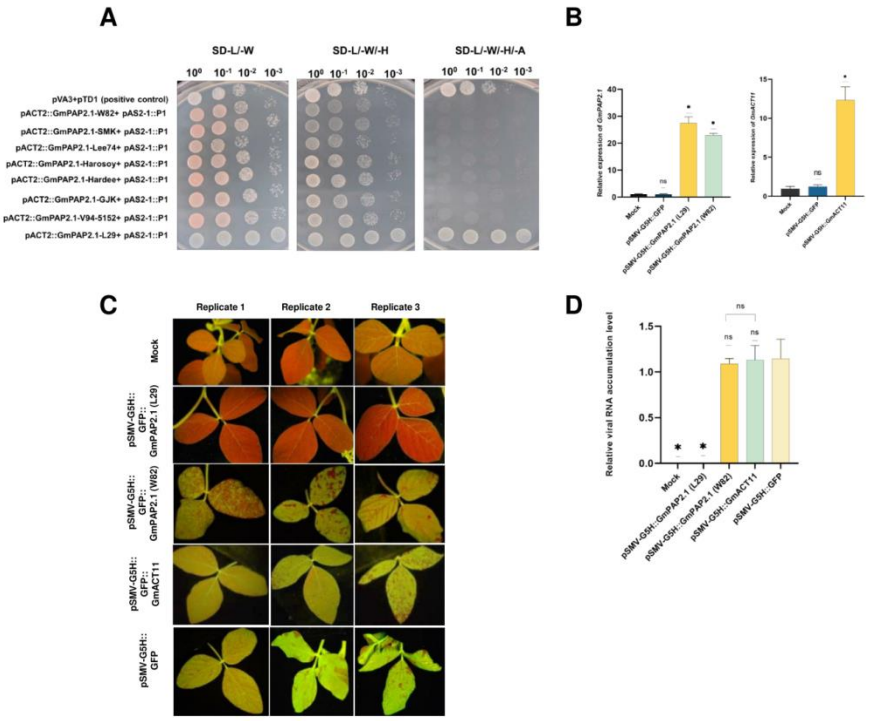


Fig. 17. A yeast two hybrid assay of the interaction between SMV-P1 protein and *GmPAP2.1* from several different soybean cultivars (A), and co-expression of pSMV-G5H and GmPAP2.1 from L29 or William 82 or with GmACT11 in soybean cultivar Lee 74 (B–D). In (A), only yeast cells expressing the combination of GmPAP2.1 from L29 and P1 could grow on the tryptophan, leucine, histidine, and adenine dropout medium. Indicating that interaction only occurs between P1 protein and GmPAP2.1 from cultivar L29. In (B), the expression level of *GmPAP2.1* from William 82 and L29 and expression level of *GmACT11*. (C) The effect of co-expression of pSMV-G5H viral vector with GmPAP2.1 from L29 or from William 82 and with GmAct11. (D) Quantification of the viral accumulation in upper non-inoculated leaves at 14 dpi. Values in (B) and (D) are means $\pm$ SD from three independent experiments. An asterisk indicates a significant difference between mock plants and *GmPAP2.1* from William 82, *GmACT11* overexpression plants or viral vector only inoculated plants, whereas "ns" indicates non-significant differences of *GmPAP2.1* from L29 overexpression plants relative to the mock plants or between *GmPAP2.1* from William 82 and *GmACT11* overexpression plants ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

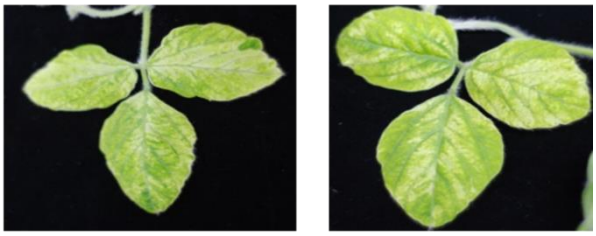
VIII. GmPAP2.1-derived resistance may not necessary for Rsv3 mediated resistance

Soybean cultivar L29 carries *Rsv3* that confers resistant to SMV-G5H (Jeong et al., 2002a; Redekar et al., 2016). Given that GmPAP2.1 was amplified from soybean cultivar L29, we evaluate requirement of GmPAP2.1 to the resistance of L29 by knocking down *GmPAP2.1* from L29 and challenge inoculated pSMV-G5H::GFP on the knock-down lines of L29, with soybean cultivar Lee 74 as a positive control. The knock-down construct of *GmPAP2.1* was generated by using the BPMV-based virus-induced gene silencing (VIGS) vector (Zhang et al., 2009). Prior challenge inoculation by pSMV-G5H::GFP, the efficiency of BPMV-mediated silencing was confirmed by observation of the bleaching symptoms in the *PDS* gene knocking down plants at 13 dpi, and decrease level of *GmPAP2.1* up to 60% in the knock-down plants compared with the one in the healthy mock (Fig. 18B). Following challenge inoculation by pSMV-G5H::GFP, observation of the GFP expression and viral accumulation on the upper non-inoculated leaves was conducted at 14 dpi. Observation on the upper-non inoculated leaves revealed that SMV-G5H failed to infect *GmPAP2.1*-knock-down L29, unlike the one in the cultivar Lee 74 control. Both of GFP expression and viral RNAs accumulation were only observed in the Lee 74 control and not in the *GmPAP2.1*-knock-down L29 (Fig. 19A, 19B), suggests that GmPAP2.1 may not necessary for *Rsv3* mediated resistance

response against SMV-G5H.

To further confirm potential of GmPAP2.1 as a resistant factor against SMV infection without the presence of Rsv3, I co-expressed GmPAP2.1 in the non-host plant, *N. benthamiana* which lacks *Rsv3* gene, with SMV-G5H or SMV-G7H infection. Interestingly, co-expression of GmPAP2.1 and SMV-G5H or SMV-G7H inhibits SMV infection in *N. benthamiana* plants. GFP expressions that appears along with the viral replication only present on the pSMV-G5H::GFP- or pSMV-G7H::GFP-inoculated plants and absent on the GmPAP2.1 co-expression plants (Fig. 19C; pSMV-G5H::GFP::GmPAP2.1 and pSMV-G7H::GFP::GmPAP2.1). These results suggest that the GmPAP2.1 can inhibit replication of both SMV-G5H and SMV-G7H strains regardless of the presence of Rsv3 resistance factor.

A



B

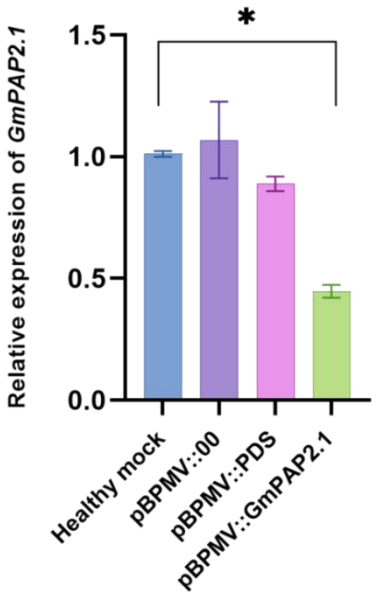


Fig. 18. Knocking-down of *GmPAP2.1* in soybean cultivar L29. (A) Bleaching symptom of PDS-knocked down plants that were used as a phenotype control. (B) Relative levels of the *GmPAP2.1* gene in plants inoculated with empty VIGS vector (pBPMV::00), knock-down *PDS* (pBPMV::PDS), and knock-down *GmPAP2.1* (pBPMV::GmPAP2.1) constructs.

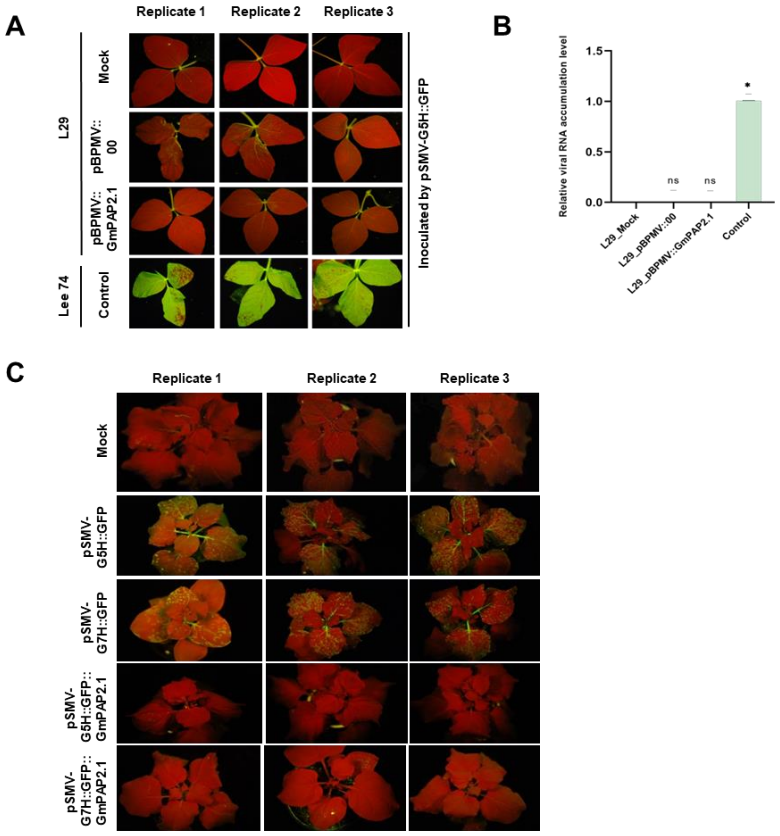


Fig. 19. Effect of knocking-down *GmPAP2.1* of L29 in Rsv3-mediated resistance and resistance response of GmPAP2.1 in *rsv3*-null *N. benthamiana* plants against SMV infections. (A) Inoculation of pSMV-G5H::GFP in the *GmPAP2.1* knock-down soybean cultivar L29 plants. Soybean cultivar Lee 74 plants were used as the positive control. Upper non-inoculated leaves were photograph at 14 dpi. The experiments were conducted in 3 replications with 3 plants in each replication. (B) Quantification of the viral accumulation in upper non-inoculated leaves at 14 dpi of pSMV-G5H::GFP. The *GmPAP2.1* knock-down line showed no virus accumulation in comparison to the positive control (B). Values in (B) are means $\pm$ SD of three independent experiments. An asterisk indicates a significant differences between positive control and mock plants, whereas "ns" indicates non-significant differences relative to the mock plants ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test). (C) Resistance response of GmPAP2.1 to SMV-G5H and SMV-G7H in *N. benthamiana*. GFP expression was only observed in the pSMV-G5H::GFP- and pSMV-G7H::GFP-inoculated plants but not in the GmPAP2.1 co-expression plants.

IX. Co-expression of GmPAP2.1 with SMV induces accumulation of SA level

To determine whether the function of GmPAP2.1 in resistance to SMV infection is related to the phytohormone such as SA and abscisic acid (ABA), I co-expressed GmPAP2.1 with SMV-G5H or SMV-G7H in soybean Lee 74 and conducted RT-qPCR with SA- and ABA-related gene-specific primers (Table 2) and with total RNAs extracted from leaves at 0, 24, and 48 hpi. The results showed a steady induction of endogenous SA-related genes in response to both SMV-G5H and SMV-G7H in the plants that co-expressed GmPAP2.1 (Fig. 20A). I also observed induction of endogenous SA-related genes (*ICS1*, *PAD4*, and *EDS1*) in response to inoculation of viruses only. There was less consistency in the results in response to inoculation with the viruses only, but all the SA-related genes were up-regulated at 48 hpi compared with the mock control. There was also variability in the detailed pattern of results over time for expression of *GmPAP2.1* only, but it was consistently up-regulated compared with the mock (Fig. 20A). In contrast, I did not observe any significant differences in the expression levels of endogenous ABA-related genes (Fig. 20B).

To determine whether the phytohormone SA is involved in GmPAP2.1-derived resistance, I generated knock-down constructs of SA- and ABA-related

genes. Two SA-related genes, *GmICS1* (*Glyma01g104100*) and *GmNPR1* (*Glyma15g127200*), and one ABA-related gene, *GmABA2* (*Glyma11g151400*), were knocking-down by transiently expressing the knock-down constructs in soybean cultivar Lee 74. At 13 dpi, the *PDS* gene knock-down plants that used as control showed bleaching symptom (Fig. 21A). I also observed stunted phenotype of SA- and ABA-related genes knock-down plants compared with the healthy and viral vector-inoculated plants (Fig. 21B). In *GmICS1* and *GmNPR1* knock-down plants, genes levels were decreased by 63% and 64%, respectively. In *GmABA2* and *GmPDS* knock-down plants, genes levels were decreased by 65% and 70%, respectively, compared to the levels in healthy plants (Fig. 21C).

Following confirmation of the knock-down level, I challenge inoculated the knock-down lines with pSMV-G5H::GFP and observed the basal level of the virus from each knock-down lines. Both GFP expression and quantification of viral RNA accumulation in upper non-inoculated leaves showed similar high level of viral RNAs in all of lines (Fig. 22A, 22B), suggesting that knock-down of those genes do not affect the basal level of the SMV-G5H in the inoculated plants.

To corroborate how GmPAP2.1-derived resistance is affected by the knock-down of SA- and/or ABA-related genes, the pSMV-

G5H::GFP::GmPAP2.1 construct was co-inoculated into upper leaves of the knock-down plants. At 14 days post-challenge inoculation of the pSMV-G5H::GFP::GmPAP2.1 construct, GFP expression was observed on the upper leaves of *GmICS1*- and *GmNPR1*-knock-down plants, and the GFP signal was stronger on *GmICS1*-knock-down plants than on *GmNPR1*-knock-down plants. In contrast, the GFP signal was not observed on the *GmABA2*-knock-down plants (Fig. 22C). Consistent with the later finding, the viral RNA accumulation was moderate in the *GmICS1*-knock-down plants, was intermediate in the *GmNPR1*-knock-down plants, and was not detected in the *GmABA2*-knock-down plants (Fig. 22D).

To evaluate the actual role of SA in the GmPAP2.1-mediated resistance against SMV, I measured the SA accumulation level during the co-expression of GmPAP2.1 and SMV-G5H or -G7H by HPLC analysis. As shown in Fig. 10E, SA accumulation level in the GmPAP2.1 and SMV-G5H or -G7H co-expression plants are 2.716 or 2.422 μg/g fresh weight (FW) of tissue samples, respectively. These accumulations are 20 and 18 folds higher compared with the one in the healthy control (0.137 μg/g FW). I also observed increased accumulation level of SA from the GmPAP2.1 only overexpression (GmPAP2.1) or from SMV infections (pSMV-G5H and pSMV-G7H) as compare to the healthy plants. However, the increases are lower compared with the one in the co-expression

(pSMV-G5H::GmPAP2.1 and pSMV-G7H::GmPAP2.1) plants (Fig. 22E). Together, these data support the hypothesis that SA plays a critical role in the GmPAP2.1-mediated resistance response against SMV infections.

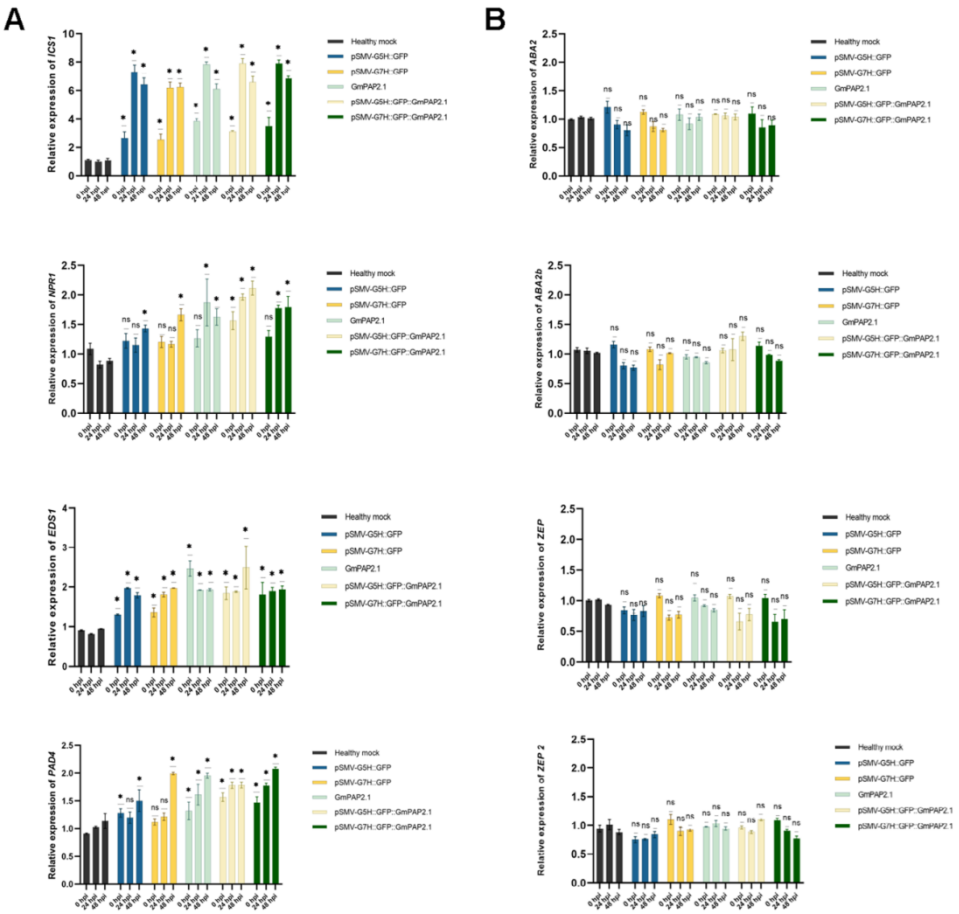


Fig. 20. The expressions of endogenous SA-related genes (A) and endogenous ABA-related genes (B) in Lee 74. Soybean cultivar Lee 74 was mock inoculated or inoculated with *GmPAP2.1* expression clone, or inoculated with pSMV-G5H::GFP with or without the *GmPAP2.1* expression clone or with pSMV-G7H::GFP with or without the *GmPAP2.1* expression clone. Expression was evaluated at 0, 24, and 48 hpi using RT-qPCR. Values are means ± SD of three independent experiments. An asterisk indicates a significant differences relative to the mock plants, whereas "ns" indicates non-significant differences relative to the mock plants ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

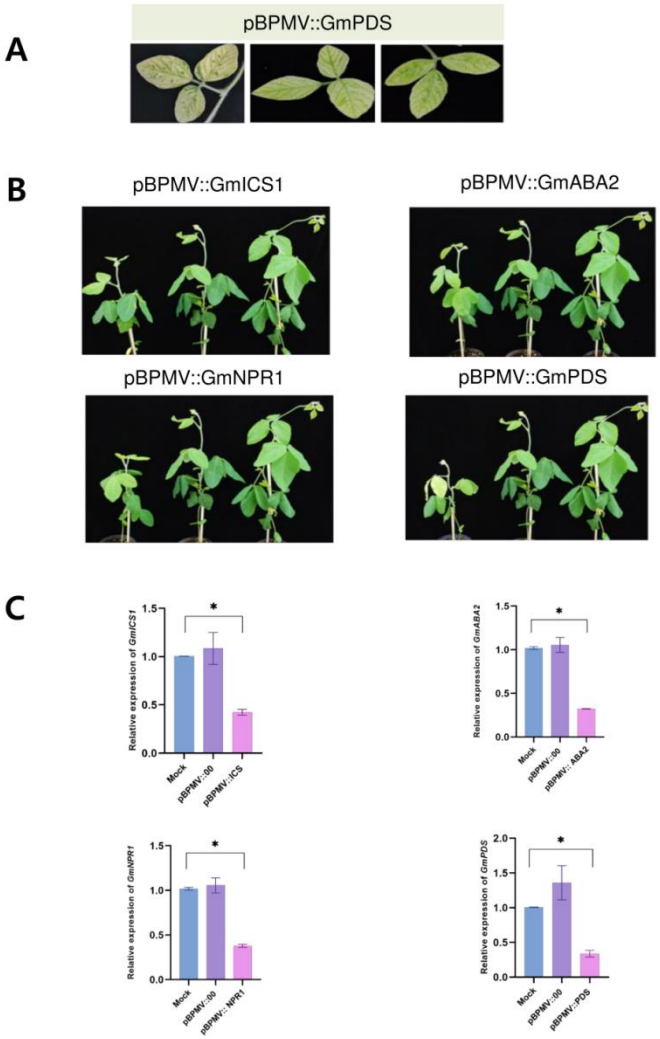


Fig. 21. Knocking-down of SA- and ABA-related genes in soybean cultivar Lee 74. (A) Bleaching symptom of PDS-silenced plants was evident at 13 dpi. (B) Silenced plants (right) were stunted compared to plants inoculated with the empty VIGS vector (pBPMV, middle) and healthy plants (right). (C) Levels of genes in the knock-down plants. The levels of *GmICS1* or *GmNPR1* decreased up to 63% and 64% while levels of *GmABA2* and *GmPDS* decreased by up to 65% and 70%, respectively.

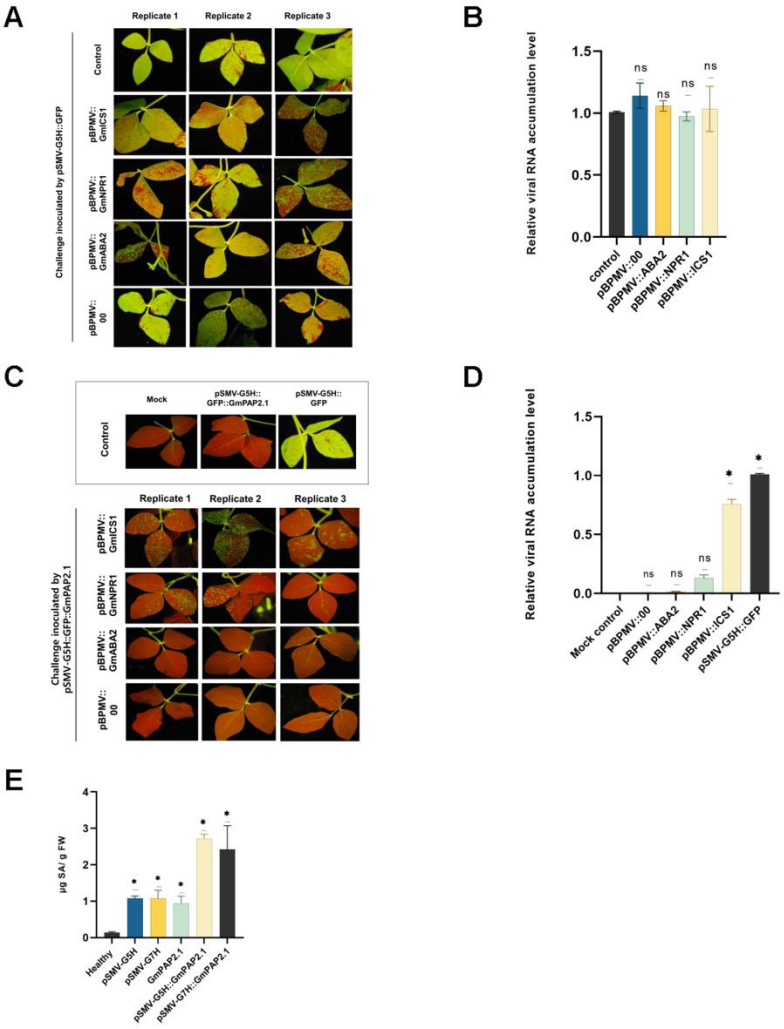


Fig. 22. Effects of co-inoculation of pSMV-G5H::GFP without (A–B) or with (C–D) GmPAP2.1 on Lee 74 plants in which *GmICS1*, *GmNPR1*, or *GmABA2* genes were transiently knock-down. (A) GFP expression was observed in all of inoculated plants. (B) Quantifying of basal viral accumulation level in upper non-inoculated leaves at 14 dpi. (C) Co-expression of *GmPAP2.1* and SMV-G5H in the transiently knock-down Lee 74. (D) Quantifying of viral accumulation level in upper non-inoculated leaves at 14 days post challenge inoculation on knocking-down lines by pSMV-G5H::GmPAP2.1. GFP expressions in (C) were evident and the virus was detected when *GmICS1* and *GmNPR1* were knock-down but not when *GmABA2*

was knock-down. (E) The active salicylic acid level from the healthy plant, virus only inoculated plant, GmPAP2.1 only overexpression plants, and GmPAP2.1 overexpression plant with the present of viruses were measured by HPLC analysis. Values in (B, D and E) are means ± SD of three independent experiments. Asterisks indicate differences between samples relative to the mock plants or healthy plant, where “ns” indicates non-significant differences relative to mock plants ($p<0.05$, according to ANOVA with Tukey’s HSD post Hoc Test).

DISCUSSION

In this study, I demonstrated that a novel purple acid phosphatase from soybean cultivar L29, GmPAP2.1, inhibited systemic movement of SMV-G5H and delayed the infection of SMV-G7H in soybean cultivar Lee 74 (Fig. 10A–10E). In the soybean cultivar L29–SMV pathosystem, a key *R* gene (*Rsv3*) that confers resistance to SMV-G5H specifically recognizes the CI protein of the virus as an effector, resulting in ER (Seo et al., 2014). In the current study, I predicted that GmPAP2.1 works in similar manner by recognizing a specific SMV protein, which results in a resistance response. Using Y2H, ß-galactosidase, and Co-IP assays, I found that GmPAP2.1 interacts only with the P1 protein of SMV (Fig. 12A–12C).

The P1 protein is predicted to be involved in the suppression of host defense and thereby facilitates virus adaptation to a wide range of host species (Maliogka et al., 2012; Valli et al., 2007; Widyasari et al., 2020). The P1 protein from SMV-P, an SMV that was isolated from *P. ternata*, was previously found to interact strongly with the host Rieske Fe/S protein, a cytochrome *b6/f* complex that is involved in electron transfer during photosynthesis in the chloroplast, and interacts moderately with Rieske Fe/S protein of *G. max* cv. Xudou 1 (Shi et al., 2007); this interaction promotes symptom development and

host adaptation of SMV. Unlike the interaction of P1 with Rieske Fe/S, which induces viral adaptation and symptom development in *P. ternata* (Shi et al., 2007), interaction between P1 and GmPAP2.1 in the current study, however, induced a strong defense response to SMV in soybean cultivar Lee 74. Considering the antagonistic results of the interaction between P1 and GmPAP2.1 or Rieske Fe/S, I conducted Co-IP assay to evaluate possibility of competition to bind to P1 between these two proteins. Co-IP assay of these three proteins did not show a noticeable competition between GmPAP2.1 and GmRieske Fe/S in interacting with P1 protein (Fig. 12C). The relative band densities of GmPAP2.1 that was precipitated with the presence of P1 or GmRieske Fe/S that was precipitated with the presence of P1 are not far different from bands of samples that were precipitated with the presence of three proteins (Fig. 12C, values under each band).

Nevertheless, the results of Co-IP assays showed that the interaction between P1 and GmPAP2.1 was stronger than that with GmRieske Fe/S, and this was consistent with a previous report where P1 of SMV-S (SMV isolated from soybean) was found to interact weakly with the *G. max* host Rieske Fe/S protein (Shi et al., 2007). Accordingly, I conclude that because GmPAP2.1 from L29 has a stronger interaction with P1, it prevents P1 from interacting with GmRieske Fe/S. Given that a strong interaction between P1 and Rieske Fe/S

promotes SMV infection in *P. ternate* (Shi et al., 2007),), it is possible that this competition from GmPAP2.1 contributes towards resistance in Lee 74, but further experiments are required.

Interaction analysis of P1 protein and GmPAP2.1 from 8 soybean cultivars demonstrated that only GmPAP2.1 from cultivar L29 interacts with P1 protein of SMV (Fig. 17A). Co-expression of GmPAP2.1 from William 82 in Lee 74 did not result in a resistance response like the one observed with the GmPAP2.1 from L29 against SMV-G5H (Fig. 17C, 17D). The inability of GmPAP2.1 from William 82 to provide resistance against SMV-G5H perhaps due to its failure to interact with P1 protein of SMV-G5H (Fig. 17A). In the analysis of the predicted structures, only GmPAP2.1 of L29 has one additional binding site at residue 337 (Fig. 7). The presence of the additional binding site results in the changes of protein structure, particularly in the ligand binding regions (Fig. 7, red circle). In addition, experiments with substitution and deletion mutants of GmPAP2.1 demonstrated importance of the C-terminal region, particularly aa residue at 337, for binding with P1 protein (Fig. 16C). Hence, it is possible that the presence of an additional binding site at the C-terminal region of GmPAP2.1 of L29 might provide its unique ability to form a binding complex with SMV-P1 protein.

Taking that GmPAP2.1 and P1 interact and co-localize in the chloroplast

(Fig. 12C, 12D), I then hypothesized that P1-GmPAP2.1 complex affects a mechanism that occurs in chloroplast which further result in the development of resistance response to SMV. Re-localization of GmPAP2.1 from chloroplast to nucleus mitigated the antiviral activity of GmPAP2.1 against SMV-G5H; evident by GFP expression and viral accumulation in the upper non-inoculated leaves of co-expression plants (Fig. 14B, 14C) which confirming the importance of chloroplast localization in the antiviral activity of GmPAP2.1 against SMV.

Chloroplast is a crucial organelle in the plant immunity due to its ability to synthesizing secondary metabolites and defense-related compounds including phytohormones (Kretschmer et al., 2020). SA, ABA, and jasmonic acid (JA) are phytohormones that known to be responsible for defense responses, and among them SA and JA are synthesized in chloroplasts (Seyfferth & Tsuda, 2014; Wasternack & Hause, 2013; Zhao et al., 2016). SA and JA often accumulate during plant immune responses but usually act antagonistically (Boatwright & Pajerowska-Mukhtar, 2013; Pieterse et al., 2012). The SA pathway is a major signal pathway for effector-triggered immunity and systemic acquired resistance (SAR). The SA signaling pathway is effective against biotrophic pathogens that produce effectors early during infection (Fu & Dong, 2013; Pieterse et al., 2012), whereas the JA pathway

helps defend against necrotrophic pathogens by regulating the activation of induced systemic resistance (Alazem & Lin, 2015; Ishiga et al., 2017; Pieterse et al., 2012; Robert-Seilaniantz et al., 2011). Because plant viruses are biotrophic pathogens, I focusing my study on the relationships between GmPAP2.1 and SA in order to understand the defense response against SMV.

Examination of SA-related genes following inoculation with SMV-G5H and SMV-G7H resulted in induction of *GmICS1*, *GmEDS1*, and *GmPAD4* (Fig. 20A). Correspondingly, induction of *GmICS1*, *GmEDS1*, *GmPAD4* and *GmNPR1* were observed in the *GmPAP2.1* overexpressing plants, while co-expression of *GmPAP2.1* and SMV-G5H or SMV-G7H resulted in significant induction of *GmICS1*, *GmEDS1*, *GmPAD4* and *GmNPR1*. Both *ICS1* and *NPR1* encode critical enzymes for the regulation of the SA pathway (Seyfferth & Tsuda, 2014). NPR1 functions as a transcriptional co-activator, and its transcriptional activation causes SA to activate the expression of key immune regulators. Mutation of NPR1 has been shown to abolish its ability to promote SA-induced expression of defense genes (Ding et al., 2018). ICS1 is a critical enzyme in the pathogen-induced SA biosynthesis pathway (Garcion et al., 2008; Seyfferth & Tsuda, 2014). In the double mutant *sard1-1 cbp60g-1*, SA accumulation and *ICS1* induction are blocked during pathogen infection of *Arabidopsis*, resulting in the loss of SAR (Zhang et al., 2010). Taking this into

account, I knocked down *GmNPR1* and *GmICS1* in the susceptible cultivar Lee 74 and this compromised the resistance to SMV-G5H derived from GmPAP2.1 (Fig. 22C, 22D). Evaluation on the SA accumulation level upon the virus infection or GmPAP2.1 expression demonstrated high accumulations of SA in the GmPAP2.1 co-expression with SMV (Fig. 22E). However, only much smaller increases in concentration were observed in plants where SMV and GmPAP2.1 were expressed alone. These results imply that accumulation of SA is induced during SMV infection in Lee 74, but insufficient concentrations are present to overcome viral replication and infection. In contrast, co-expression with GmPAP2.1 increases the SA accumulation sufficiently to enable it to activate SAR, which then inhibits the SMV infection.

In summary, I conclude that GmPAP2.1 from the soybean cultivar L29 binds with the P1 protein of SMP as a result of a unique structural change at aa residue 337 in the C-terminal region. This results in induction of genes that regulate the SA synthesis pathway, triggering increased production and accumulation of SA that in turn activates systemic acquired resistance in response to the presence of SMV infection. Hence, overexpression of GmPAP2.1 from L29 has the potential to significantly enhance resistance to SMV in susceptible soybean cultivars.

The GmPAP2.1 that used in this study was amplified from soybean

cultivar L29 that confers extreme resistance specific to SMV strain G5H due to the present of Rsv3. My study, however, demonstrated that overexpression of GmPAP2.1 (L29) on the susceptible cultivar confers resistance to both strains of SMV-G5H and G7H (Fig. 10A–10E). The difference in the strain specificity of these two resistance factors may due to the distinct response of Rsv3 and GmPAP2.1 upon SMV G5H and G7H infection. Transcription level of *GmPAP2.1* in L29 is increased upon infection of both strains SMV G5H or G7H (Fig. 2). *GmPAP2.1* expression level was continuously upregulated upon infection of SMV-G5H (Observations at 8, 24, and 54 hpi). Although there was less consistency in the results in response to infection with the SMV-G7H, *GmPAP2.1* was also up-regulated at 8 and 54 hpi compared with the mock control (Fig. 2). Moreover, examination of the expression level of the endogenous SA-related genes and evaluation of SA accumulation showed consistent induction of endogenous SA-related genes (Fig. 20A) and high accumulation of SA (Fig. 22E) in response to both SMV-G5H and SMV-G7H in the Lee 74 plants that co-expressed GmPAP2.1 (Fig. 20A) which lead to the resistance response to both strains. Whilst in Rsv3 response to SMV infection, resistance is obtained through rapid accumulation of callose which limits SMV-G5H movement in early time point, evident by a rapid induction of several genes involved in ABA biosynthesis at 8 hpi in response to G5H but not to G7H

(Alazem et al., 2018). This induction, however, declined at 24 hpi and the expression of several of these genes was reduced at 54 hpi (Alazem et al., 2018). Taking these together, I assumed that Rsv3 is work in the early infection of SMV G5H and arrest it on the inoculation sites, while GmPAP2.1 activity works continuously during early and later infection stages of SMV.

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Chapter 3

A heat shock protein 70 family member, BIP 2, is necessary for soybean mosaic virus infection in *Nicotiana benthamiana*

ABSTRACT

Soybean mosaic virus (SMV) is one of the most prevalent plant virus which has narrow host range with soybean as the main host plant. In my study, I observed systemic infection of SMV strain G5H and G7H in *Nicotiana benthamiana* following mechanical inoculation by soybean infected tissue and/or *Agro*-inoculation. Analysis of the transcription level of several host factors demonstrated upregulation of endoplasmic reticulum-associated host factors, i.e., *HSP 70-a*¸ *BIP 1* and *BIP 2* following infection by SMV. Knock-down of *BIP 2* impair SMV infection without causing any significant disruption on plants vigor and vitality. Subcellular localization assay demonstrated co-localization of BIP 2 and SMV-NIb on the ER-like compartment. In addition, *in vivo* analysis by yeast two hybrids corroborates the interaction between BIP 2 and NIb protein. Taking all of these results into accounts, I proposed that SMV may recruits the ER-related host factor, BIP 2, into viral replication complex to assist and promote protein maturation and viral replication.

Key words: soybean mosaic virus, *Nicotiana benthamiana*, endoplasmic reticulum, replication, host factor.

INTRODUCTION

Heat shock protein 70 (HSP70) family is a group of molecular chaperon that highly conserved in many organisms, including eubacteria, plant, animal, and human (Chen et al., 2008; Daugaard et al., 2007). HSP70 proteins plays a crucial role to assist newly synthesized protein to fold into unique three-dimensional structures in order to become functionally active, to prevent protein misfolding or aggregation in the crowed environmental cell, to assist in the transport of precursor proteins into organelles, and to help target damaged proteins for degradation (Hartl & Hayer-Hartl, 2002; Sung et al., 2001). The HSP70 family proteins are also well-known to play pivotal roles in response to the various stresses including heat, drought, acidity, and salt stresses (Anaraki et al., 2018; Duan et al., 2011; Montero-Barrientos et al., 2010; Yu et al., 2015).

HSP70s have two major functional domains, which are an ATPAse nucleotide binding domain (NBD) with size approximately 40 KDa which located in the N-terminal region of the protein and a substrate binding domain (SBD) in the C-terminal region of the protein with size approximately around 25 KDa. These two domains are separated by a linker region that enables allosteric communication between two structured domains (English et al., 2017;

Sung et al., 2001; Usman et al., 2017). The C-terminal region of HSP70s containing a protein-binding EEVD motif that enable it to bind to co-chaperon or other HSPs (Chen et al., 2008; Mayer & Bukau, 2005). Each of HSP70 member has a cellular localization signal responsible to localize the protein into specific cell's compartment such as cytosol, endoplasmic reticulum (ER), mitochondria, and plastids (Daugaard et al., 2007).

HSP70 family proteins in human contains at least eight homologous chaperone proteins that distribute in ER, mitochondria, cytosol, or nucleus (Daugaard et al., 2007). Around 61 putative HSP70 genes were reported from soybean genome, which distributed unequally on 17 of the 20 chromosomes and were differentially expressed in a tissue-specific expression pattern (Zhang et al., 2015). In model plants such as *Arabidopsis thaliana,* at least 18 genes encoding member of the HSP70 family proteins were reported (Lin et al., 2001), and at least ten genes have been reported in *Nicotiana benthamiana* (Kanzaki et al., 2003; Yang et al., 2017).

In the interplay between plant viruses and host factors, the functions of HSP70s are beyond the folding of viral protein. Infection of chinese wheat mosaic furovirus (CWMV) in *N. benthamiana* or wheat (Yang et al., 2017) and tobacco mosaic virus in *N. benthamiana* (Chen et al., 2008) positively induced

expression level of *HSP70* gene, while transient knockdown or treatment using HSP70 inhibitor, quercetin, reduced viral accumulation (Chen et al., 2008; Yang et al., 2017). HSP70 protein member have also been reported to play a role in protein maturation, multiplication, virion assembly and movement of plant viruses as well as to maintain host physiology (Alzhanova et al., 2001; Gorovits et al., 2013; Hafrén et al., 2010; Mine et al., 2012).

As one of the most devastating pathogen that cause huge economic loses of worldwide soybean production for each year, management of soybean mosaic virus (SMV) has become a priority in many soybean growing areas (Hajimorad et al., 2018; Wrather et al., 2001). So far, management of SMV infection is mostly depending on the cultivation of the resistant cultivar (Shakiba et al., 2012). With current rapid development of the agricultural technology, however, generation of transgenic plants which deploy concept of viral pathogen–host factors interaction such as by modification of the host factors that are necessary for viral replication may help to minimizing losses due to the SMV outbreak on the growing area. Nevertheless, report about host factors that may participate and promote infection cycle of SMV remains inadequate. In my study, I observed the regulation of HSP70 family proteins, i.e., BIP 1, BIP 2, and HSP70-a, by SMV infection while knockdown of those

genes impaired SMV infection in *N. benthamiana*. Subcellular co-localization of SMV NIb protein and BIP 2 protein (a HSP70 protein member that exist in the ER) and *in vitro* interaction between these two proteins imply the possibility of BIP2 being recruited by SMV from ER into viral replication complex to promotes protein maturation and viral replication.

MATERIALS AND METHODS

I. Plants growth condition and virus inoculation

N. benthamiana plants were grown in a growth chamber at 25 °C, with 16h light/8h dark photoperiod, and 65% relative humidity. Virus inoculation was conducted in two methods; mechanical inoculation using SMV strain G5H and G7H that expressing green fluorescence protein (GFP) signal infected soybean leaves tissues and agroinfiltration using infectious plasmids clones of SMV-G5H and -G7H that expressing GFP signal (Seo et al., 2016). Systemic infection was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) with SMV capsid protein (CP) specific primer pairs and GFP expression that was photographed under UV-light.

II. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNAs were extracted from the upper non-inoculated leaves by the RNAiSO plus reagent method (TaKaRa, Japan). Equal amounts of total RNAs (2 µg) were used for cDNA synthesis using GoScript ™ Reverse Transcriptase (Promega) and Oligo$(dT)_{15}$ primer. Technical triplicate RT-qPCR reactions were prepared using IQ™ SYBR Green Supermix (Bio-Rad) according to the

manufacturer's instruction in a CFX384 Real-Time PCR detection system (Bio-Rad). Each reaction mix (10 µl) consisted of 20 ng of cDNA, 5 µl of 2 X IQ^{TM} SYBR Green Supermix, and 10 pmoles of each primers. The qPCR was performed as follows: 95 ^{o}C for 3 min, followed by 40 cycles at 95 ^{o}C for 10 s, 59 ^{o}C for 30 s and melting curve data was obtained by increasing the temperature from 55 ^{o}C to 95 ^{o}C for 1 s/step. Two endogenous reference genes were used as reference genes to normalize qPCR results. *NbActin* and *Nb-β-Tubulin* (Baek et al., 2017) were used to normalize qPCR results. Melt curve analysis was carried out using the Bio-Rad CFX manager V1.6.541.1028 software (Bio-Rad). Each sample was evaluated in at least three independent experiments, including three technical replicates. The primer sets used for qPCR are show in Table 2.

III. Gene knock-down construct and agroinfiltration

Knock-down of HSP70 family protein (HSP70, BIP 1, and BIP 2) were conducted by using tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS). Each of target gene was partially amplified (300 bp) from *N. benthamiana* using primer pairs that were listed on Table 1. The products were digested using *Xba*I and *Bam*HI restriction enzymes and then inserted into the open reading frame of the RNA 2-encoded polyprotein of TRV (Bachan &

Dinesh-Kumar, 2012). The resulting constructs were later transformed into electro-competent cells of *Agrobacterium tumefaciens* GV3101 (GoldBio) and were transiently expressed on *N. benthamiana* by agroinfiltration as described previously (Bendahmane et al., 1999) with some minor modification.

IV. Assay of viral infection following knockdown of NbHSP70 family proteins

To assess SMV accumulation and movement following knockdown of HSP70 family proteins, an infectious clone of pSMV-G5H::GFP (SMV-G5H that expressing GFP protein) was agroinfiltrated into second upper leaves of *N. benthamiana* that emerged 10 days following knockdown of each target gene. A wild type *N. benthamiana* plants that infected by pSMV-G5H::GFP were used as positive control (viral infected plants). The fluorescence of GFP in plants was visualized at 14 days post infiltration or inoculation (dpi) under UV-lamp and photograph by digital SLR camera (NIKON D80).

V. Construction of expression clone for transient expression in *N. benthamiana*

BIP 2 gene was amplified using specific primer pair (Table 1). PCR-amplified fragments were digested with *Xba*I and *Bam*HI restriction enzyme, purified using Nucleospin PCR clean up and gel extraction (Macherey-Nagel),

and inserted into pBIN61-binary vector with mCherry tag to help the visualization of the BIP 2 in the *N.benthamiana* cell. The construct was transformed into *Agrobacterium* strain GV3101 competent cell and agro-infiltrated into *N. benthamiana*. At 2 dpi, the mCherry fluorescence signal was observed under fluorescence microscope (Leica).

VI. Subcellular localization analysis in epidermal cells of *N. benthamiana*

The transient expression vector pBin61 (Bendahmane et al., 2002) with mCherry tagging was used to express BIP 2 and pSITE binary vector (Chakrabarty et al., 2007) with GFP tagging was used to express SMV-NIb in *N. benthamiana*. The construct was introduced into *Agrobacterium* strain GV3101 and agroinfiltrated into the leaves of *N. benthamiana* as described previously (Bendahmane et al., 1999) with minor modification. At 2 dpi, fluorescence microscopy (emission at 509 nm and 610nm, exposure 400 ms) was used to assess GFP and mCherry expression in the epidermal cell. Co-localization between proteins were evaluated by performing Pearson's correlation coefficient (PCC), Mander's overlap coefficient (MOC), and cytofluogram in ImageJ software (Rueden et al., 2017).

VII. Yeast two-hybrid and beta-galactosidase assays

For yeast two-hybrid (Y2H) assays, *BIP 2* was cloned into pACT2 (AD), and SMV-G5H proteins were cloned into pAS2-1 (BD). The construct with the combination of AD and BD was then co-transformed into yeast strain AH109, which was grown on plates lacking leucine and tryptophan (SD-Leu/-Trp) for 2 days at 30 ^{o}C. Single colonies were selected and grown on SD-Trp/Leu broth medium into 0.5 at OD_{600} and transferred on SD-Leu/-Trp, or SD-His/-Leu/-Trp, or SD-His/-Leu/-Trp/-Ade agar medium in serial dilution 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} for 2 days at 30 ^{o}C.

VIII. Phylogenetic analysis

Since the annotation of *N. benthamiana* genome is not complete, I searched the sequence of genes that encoded HSP70 proteins in *Glycine max* using their homology in *A. thaliana* database. The obtained sequences further were aligned and compared with the sequence of genes that encodes HSP70 proteins in *N. benthmiana* that used in this study. The sequence of hits and their percentage of similarity were used to construct a phylogenetic tree using neighbor-joining and bootstrap methods in MEGA X (Kumar et al., 2018).

IX. Statistical analysis

Experiments were conducted at least three times (replicates) with three individual plants for each replicate. Means of treatments were compared using ANOVA with Tukey's HSD post Hoc Test in SPSS. The graphs were generated using GraphPad Prism (USA). The differences were considered significant at $p < 0.05$.

Table 1 List of primers used for amplification of inserts.

No	Primer name	Forward (5'→3') [b]	Reverse (5'→3')	Purpose
1	BIP 2	GCTCTAGAatggctggctatttga	CGGGATCCcagctcatcatggcc	Localization
2	NIb	CACCgggagaaaggaaagatgg	TTAttgtaaggacactgattc	Localization (entry to the donor vector)
3	BIP 2	gcGGCCNNNNNGGCCatggctggctatttga	gcGGCCNNNNNGGCCcagctcatcatggcc	Y2H
4	HSP70-a	GCTCTAGAcaggtagctatgaaccctatc	CGGGATCCatcctttgtggcctga	TRV-based VIGS
5	BIP 1	GCTCTAGAggtgtaaatcctgatgaggca	CGGGATCCgataccattggcatcaactt	TRV-based VIGS
6	BIP 2	GCTCTAGActtagcggagagggt	CGGGATCCaacttcaaatgtgacctc	TRV-based VIGS

[b] Sequences are indicated by lowercase letters, while additions of restriction enzyme sequences are indicated by uppercase letters.

Table 2 List primers used for RT-qPCR.

No	Primer name	Forward (5'→3') [c]	Reverse (5'→3')	Accession number
1	ARF1	caaccagaggaagcagca	ccttgtttgtcgaggc	GQ859156.1
2	BI1	cattggtggcttacttacgac	gggtcaaagtcaatagcc	NM_001325559.1
3	SGT1	ggaaactggtgctagtttag	aagactcagatgcaggg	LC314287.1
4	BIP 1	ggtgtaaatcctgatgaggca	cggtgttcctaggaatcaat	X60060.1
5	BIP 2	ggtgtaaatcctgatgaggc	cggtgttcctaggaatcaa	XM_016629466.1
6	HSP70-a	ggtagctatgaaccctatc	tcacccttgtagttgacaac	KX912913.1

[c] Primers were designed with a length of about 100 bp based on the sequence of each gene according to the reference ID.

RESULTS

I. SMV strain G5H and G7H are causes infection in *N. benthamiana.*

SMV is a RNA virus that has narrow range of host plants, mostly restricted on two species plants from the same genus, i.e., *G. max* and *G. soja* (Hajimorad et al., 2018). Infection of SMV in *N. benthamiana* has been reported before, but it is limited to few strains of SMV such as SMV- SC7 (Gao et al., 2015) and SMV-N1 (Bao et al., 2020). In my study, I demonstrated infection of SMV from strain G5H and G7H in *N. benthamiana*. Both inoculation methods I used, i.e., mechanical inoculation using infected soybean tissues and agroinfiltration, resulted in the development of SMV infection in *N. benthamiana.* A GFP fluorescent that represents viral infection was detected on all treated plants (Fig. 1A). Consistently, an RT-PCR analysis using SMV-CP specific primer set and quantification of relative viral RNA accumulation confirmed the present of SMV in the leaf tissues from all of treated plants (Fig. 1B, 1C).

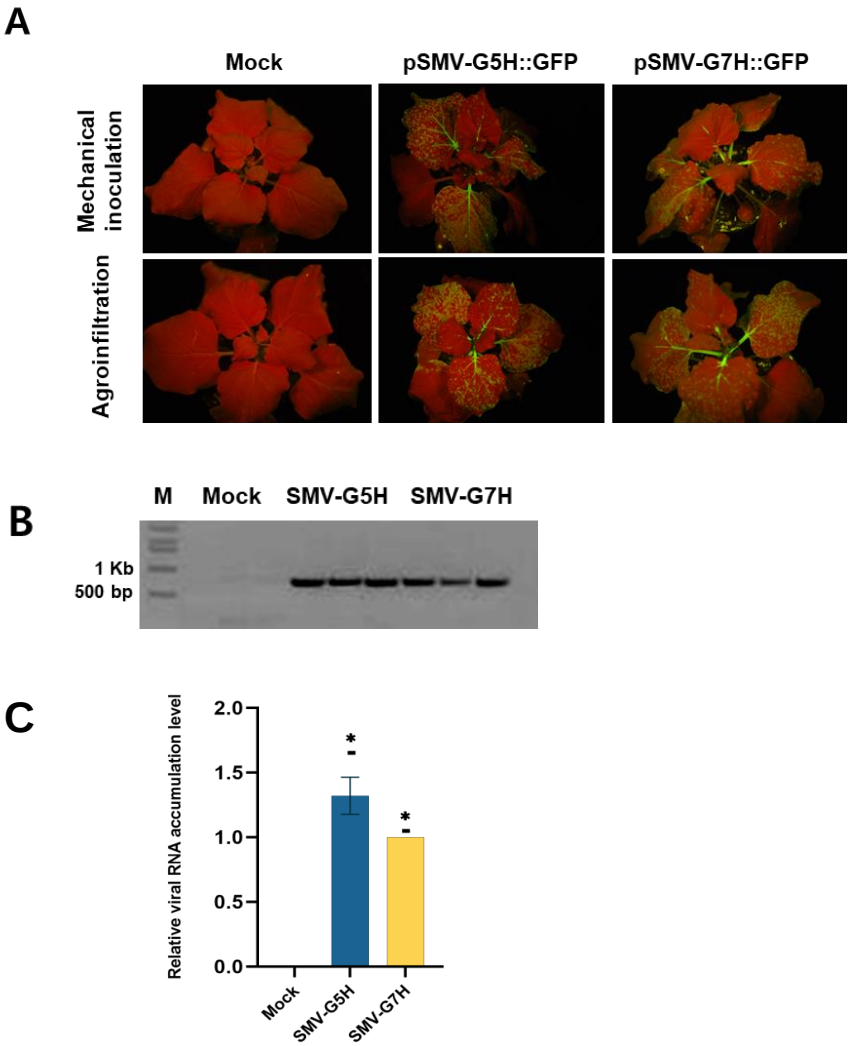

Fig. 1. Infection of SMV strains G5H and G7H in *Nicotiana benthamiana*. (A) Expression of GFP that represent SMV was detected on *N. benthamiana* that were inoculated by SMV strain G5H and G7H. (B) Detection of SMV CP gene on the *N. benthamiana* inoculated plants by RT-PCR. Target size of CP is 765 bp. (C) A quantification of relative viral accumulation level on all treated plants and mock control. Values in C are means $\pm$ SD from three independent experiments. An asterisk indicates a significant differences between SMV-inoculated plants and mock control ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

II. SMV infection in *N. benthamiana* induces upregulation of genes that encodes HSP70 family member.

Considering that *N. benthamiana* is not a natural host for soybean mosaic virus, I assumed that SMV infection in *N. benthamiana* must be related to the interaction between SMV protein(s) and host factor(s) that may promote SMV infection. To confirm this hypothesis, I conducted qPCR to analyze the accumulation of several host factors which previously have been reported necessary in pathogens infection (Garcia-Ruiz, 2018; Hafrén et al., 2010; van Schie & Takken, 2014). The qPCR was performed using cDNA that was synthesized from total RNA of *N. benthamiana* infected by SMV strains G5H or G7H at 14 dpi. Given that annotation of the genome sequence for *N. benthamiana* is incomplete, primers that were designed for qPCR were designed based on the sequence of its homolog from *N. tabacum* (primer sequences: Table 2, homolog genes from *N. tabacum*: Table 3). Analysis of the expression profile of selected genes demonstrated upregulation of gene that encodes BI1 up to 1.8 fold upon infection of SMV strain G5H and strain G7H (Fig. 2A). While the genes that encodes HSP70 family proteins; X60060.1, XM_016629466.1, and XM_016629466.1 were upregulated up to 3.5, 5.2, and 9 folds, respectively, upon infection of SMV strain G5H and up to 3, 5.8, and 4 folds, respectively, upon the infection of SMV strain G7H (Fig. 2B, 2C, and 2D), indicating that these genes were regulated at varying degree by SMV

infection. No significant expression changes were observed for the genes that encodes ARF1 and SGT1 proteins (Fig. 2E and 2F). For further investigation, among six assessed genes, I selected three most sensitive genes (accession number: X60060.1, XM_016629466.1, and XM_016629466.1, designated as *BIP 1*, *BIP 2*, and *HSP70-a* in this study for convenience).

Table 3 Host factors that may involve in the SMV pathogenesis

No	Encoded protein name	Designated name	Accession number [d]
1	Auxin response factor	ARF1	GQ859156.1
2	Bax Inhibitor	BI1	NM_001325559.1
3	For suppressor of G2 allele of skp1	SGT1	LC314287.1
4	Luminal-binding protein 1	BIP1	X60060.1
5	Luminal-binding protein 2	BIP2	XM_016629466.1
6	Heat shock protein 70	HSP70-a	KX912913.1

[d] Sequences were obtained from its homolog form *N. tabacum*

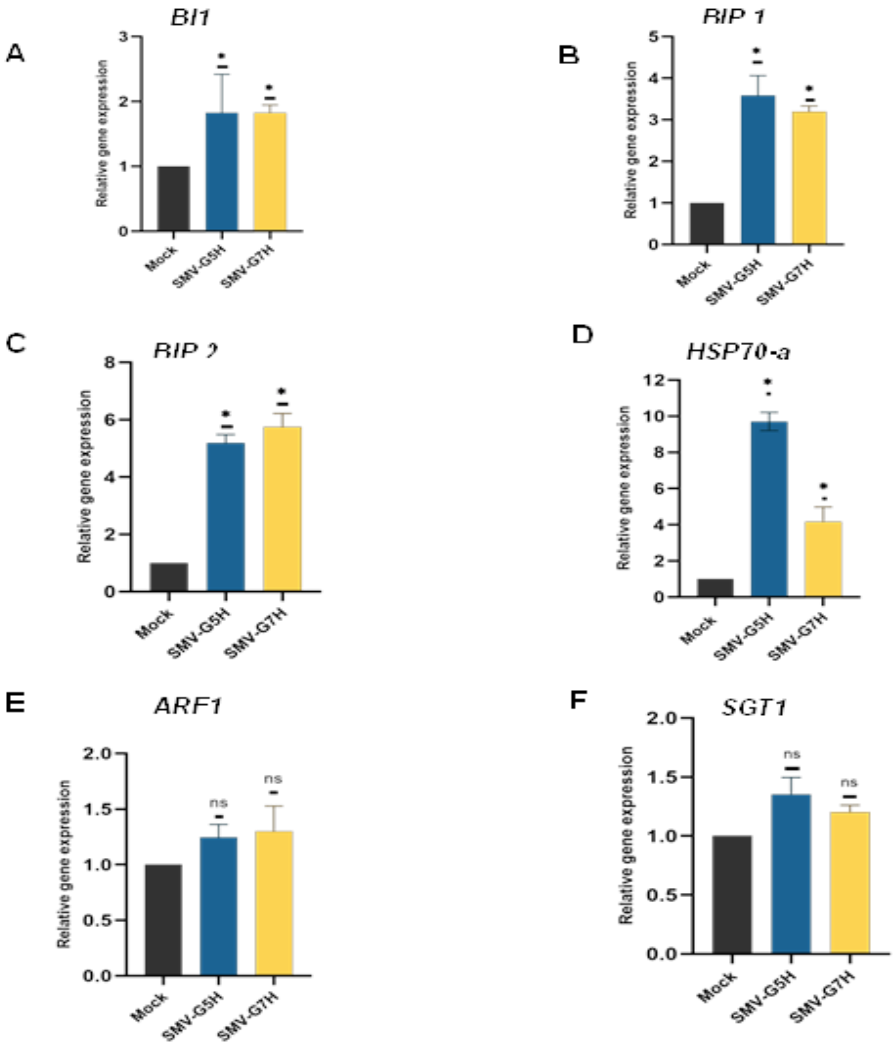

Fig. 2. Expression levels of host factors in SMV infected *N. benthamiana* plants. Values in A-F are means ± SD from three independent experiments. An "ns" indicates no significant different, and asterisk indicates a significant differences between SMV-inoculated plants and mock control. ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

III. Knockdown of HSP70 family proteins impairs SMV infection in *N. benthamiana.*

To investigate the importance of HSP70 family proteins for the SMV infection in *N. benthamiana*, I generated knockdown mutant of three genes encoding HSP70 family member, i.e., *BIP 1, BIP 2*, and *HSP70-a* that showed significantly upregulated gene expressions during SMV infection (Fig. 2B, 2C, and 2D). At 10 days post agroinoculation of knockdown construct, I observed distinct crinkle symptoms on the *BIP 1*, *BIP 2*, and *HSP70-a* knockdown lines compared to the mock. In addition, a distinct photo-bleaching symptom was observed on the *PDS* knockdown plants that used as a phenotypic control (Fig. 3A).

Following this observation, I collected samples from upper leaves of each treated plants and quantified their gene expression levels by qPCR. Quantification of the gene expression levela demonstrated down regulation of *BIP 1, BIP 2*, and *HSP70-a* genes up to 65%, 75%, and 60%, respectively, compared to the mock wild type (Fig. 3B). Subsequently, I challenge inoculated SMV strain G5H into knockdown lines and observed the SMV infection and symptom development by confirming the GFP expression on the inoculated plants which represent virus infection. The GFP fluorescence signal was detected at 14 dpi on the wild type plant infected by SMV but not on all of the

knockdown lines, suggesting that knockdown of the selected genes which encodes HSP70s family members impaired SMV infection in *N. benthamiana* (Fig. 4A). Consistently, quantification of the relative viral RNA level demonstrated accumulation of SMV RNA only in the wild type infected SMV, but none in the knockdown lines infected SMV (Fig. 4B). Taking together, these results suggest that HSP70 family members are important and may play crucial role(s) in the SMV infection cycle.

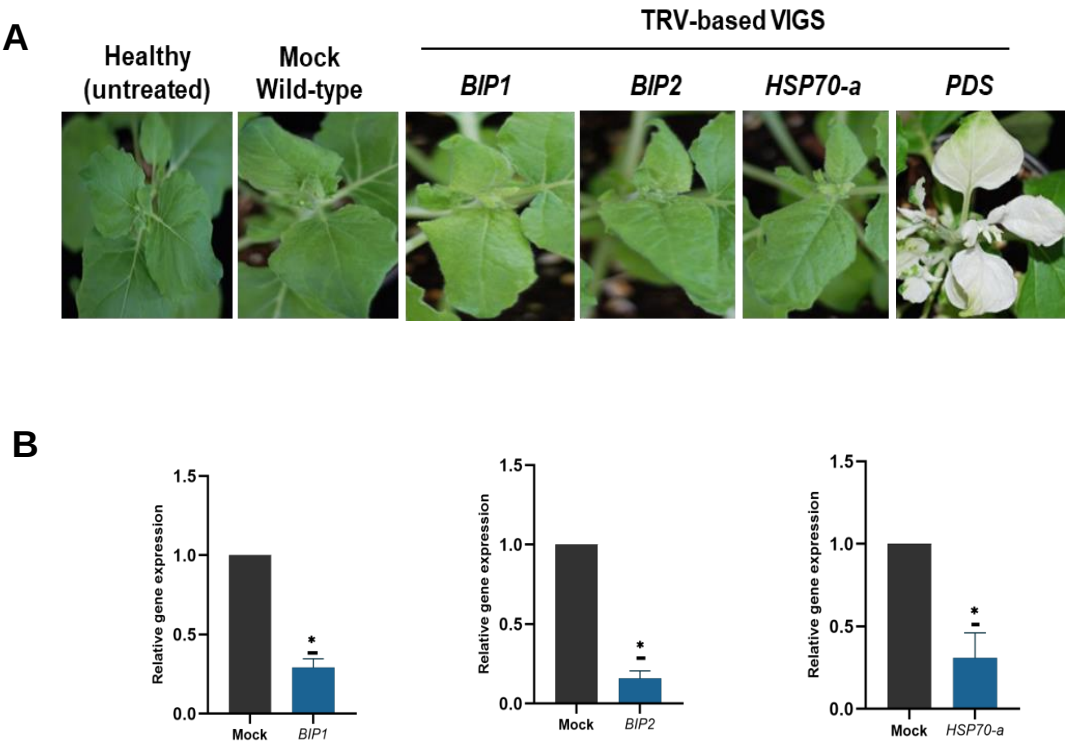

Fig. 3. Transient knockdown of genes encode HSP70 family member by TRV-based VIGS. (A) Phenotypic symptoms in *N. benthamiana* at 10 days post infiltration of TRV vector harboring a partial (300 bp) of target genes. Wild type plants that infiltrated by TRV empty vector were used as a mock control. A distinct crinkle symptom was appeared on the knockdown lines. (B) A qPCR analysis shows the expression level of *BIP 1*, *BIP 2*, and *HSP70-a* in the knockdown lines compared with the mock control. Values in B are means $\pm$ SD from three independent experiments. An asterisk indicates a significant differences between knockdown lines and mock control ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

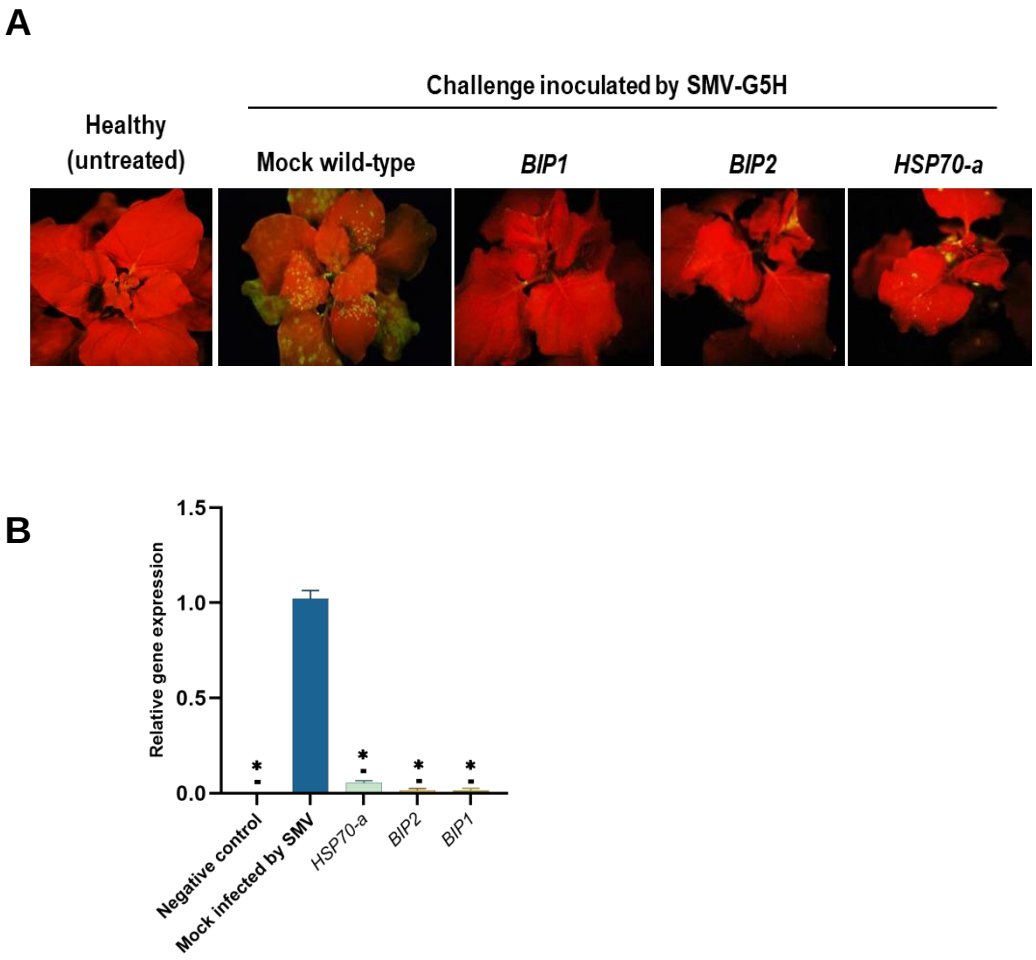

Fig. 4. Challenge inoculation of *N. benthamiana* knockdown lines by SMV strain G5H. (A) GFP expression that represent viral infection was only detected on the mock control inoculated by SMV, but not on the knockdown line inoculated by SMV. (B) A qPCR analysis shows the accumulation of relative viral RNA in the knockdown lines are significantly lower compared with the mock control. Values in B are means ± SD from three independent experiments. An asterisk indicates a significant differences between SMV-inoculated mock control and knockdown lines or negative control ($p<0.05$, according to ANOVA with Tukey's HSD post Hoc Test).

IV. NbBIP2 interacts with SMV RdRP in the endoplasmic reticulum.

In my study, three genes showed upregulation during SMV infection (Fig. 2B, 2C, and 2D) and knockdown of these genes affects SMV infectivity (Fig. 4). Nevertheless, at 21 days post SMV inoculation, the *HSP70-a* knockdown plant showed terminal death while other two knockdown lines did not show any similar phenotype (Fig. 5A). I assumed that HSP70-*a* plays important role in maintaining plant's vigor, and thus knockdown of this gene affects plants fitness. Whilst, functions of BIP 1 and BIP 2 proteins are redundant and can be replaced by their other homolog proteins to maintain plants fitness. In *A. thaliana,* BIP proteins cooperate with each other to ensure ER homeostasis. *BIP 1* and *BIP 2* are ubiquitously expressed and encode proteins that are identical to each other which later will be expressed under ER-stress condition (Maruyama et al., 2015). Considering that transcription level of *BIP 2* is higher than *BIP 1* upon SMV infection, I decided to use *BIP 2* for further investigation. I also investigate the interaction between BIP 2 and SMV proteins by Y2H analysis. Accordingly, I observed *in vivo* interaction between BIP 2 and NIb or NIa protein (Fig. 5B).

BIP protein has also been reported to possess an ER retrieval motif in its C-terminal region (Jin et al., 2017). Subsequently, I analyzed the protein prediction structure of BIP 2 of *N. benthamiana* and confirmed the presence of

HDEL motif at the end of C-terminal region (Fig. 6A). Considering that NIb protein from SMV is confirmed to be interacted with BIP 2 by Y2H (Fig. 5B), I also assessed the protein prediction structure of NIb protein and confirmed presence of KKXX motifs (dilysine retrieval motif) that might be responsible for retrieval of ER membrane proteins to and from the golgi aparatus (Stornaiuolo et al., 2003) (Fig. 6B). Following these analyses, I conducted subcellular co-localization assay of BIP 2 and NIb proteins. Observations of the mCherry fluorescence signal that represent BIP 2 protein showed distribution of BIP 2 on the ER-like compartment (Fig. 7). Correspondingly, the GFP fluorescence signal that represent NIb protein is also observed on the web-like line that extended from nucleus to the cytoplasm which identified as ER (Fig. 7). To corroborate these results, I performed correlation analyses by Pearson's correlation coefficient (PCC) and Mander's overlap coefficient (MOC) and confirmed that both proteins are strongly correlated. Taking together, these results suggested the possibility of BIP 2 being recruited by SMV from ER to assist viral replication processes and viral protein maturation.

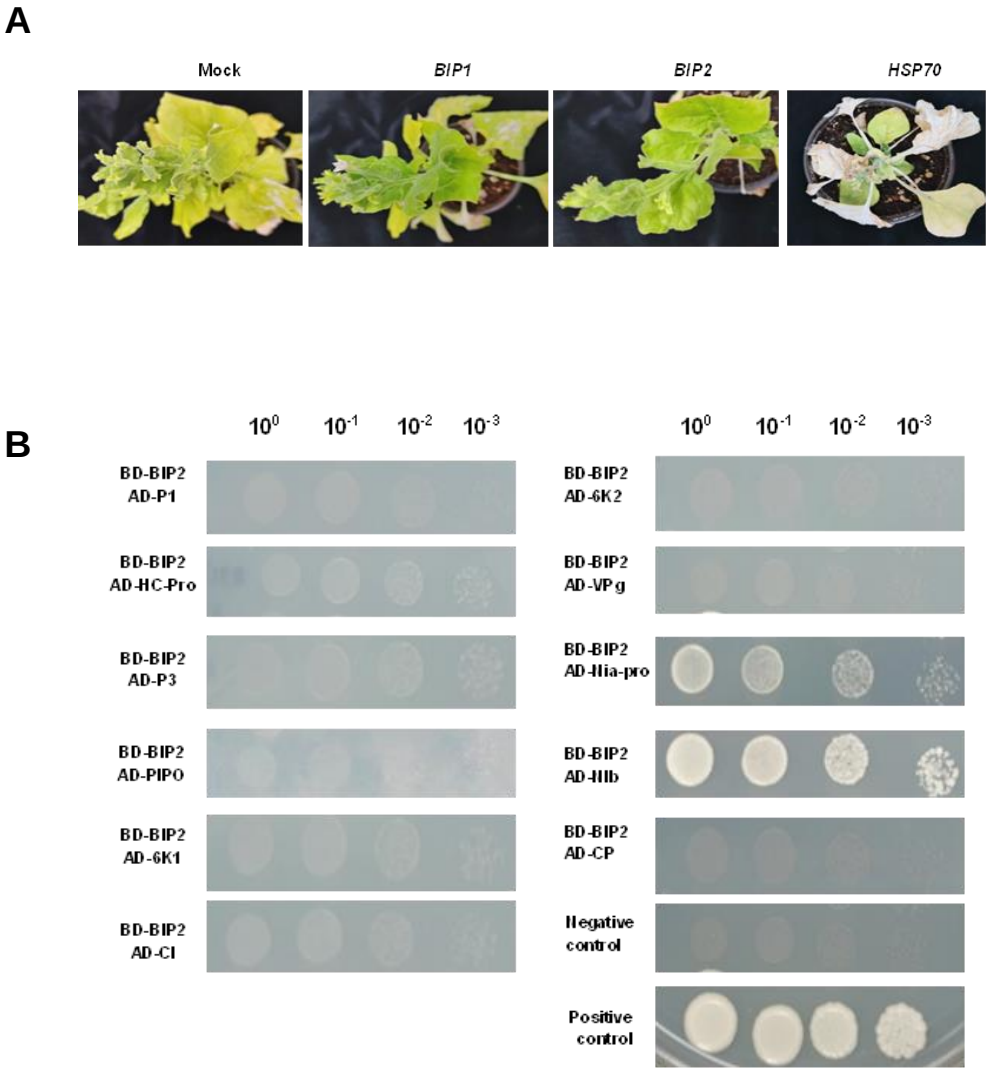

Fig. 5. Phenotype of *N. benthamiana* knockdown lines following challenge inoculation by SMV at 21 dpi and *in vivo* interaction of BIP 2 protein and SMV proteins. (A) Knockdown of *HSP70-a* caused terminal death of *N. benthamiana* (observation at 21 dpi). (B) Y2H analysis of BIP 2 and SMV proteins showed *in vivo* interaction between BIP 2 of *N. benthamiana* and SMV NIb or NIa protein.

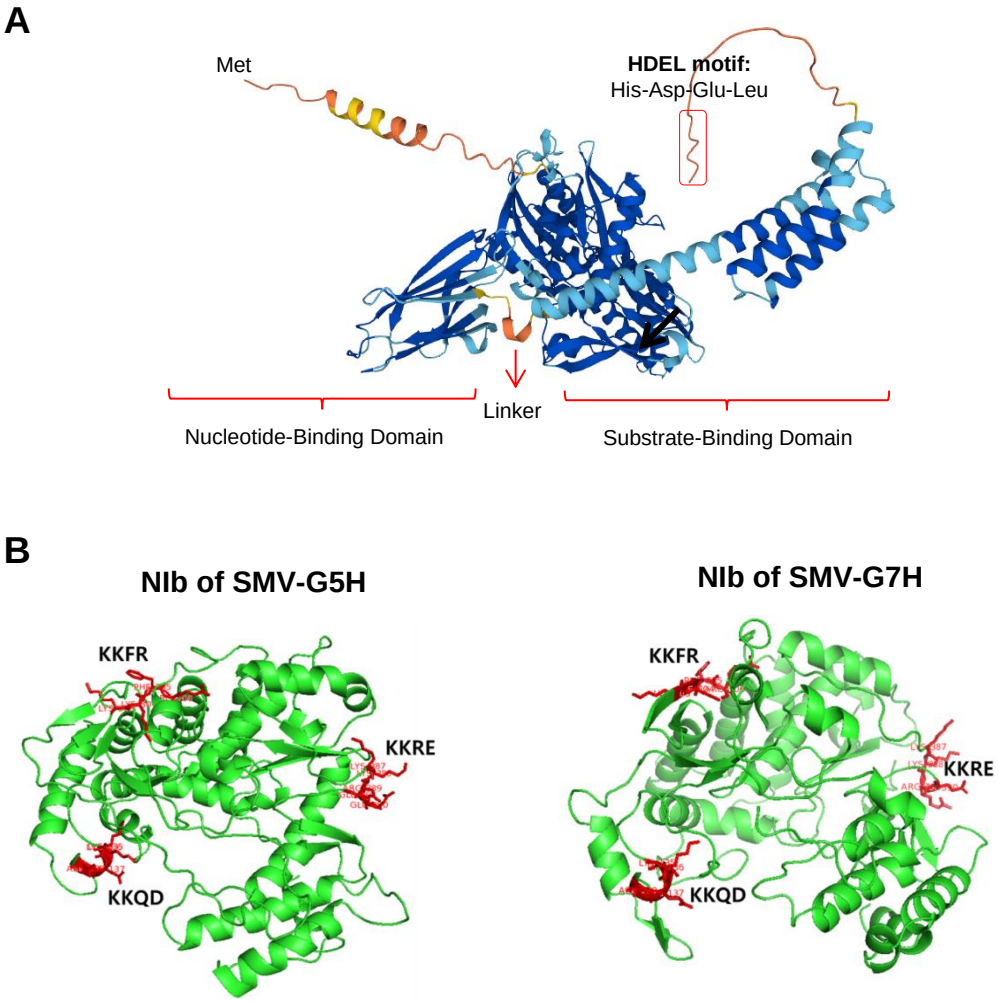

Fig. 6. Prediction protein structure of BIP 2 and SMV-NIb. (A) Prediction protein structure of BIP 2 with HDEL motif at the end of C terminal. (B) Prediction protein structure of NIb protein of SMV strain G5H and G7H with KKXX motif (dilysine retrieval motif) that showed in red color.

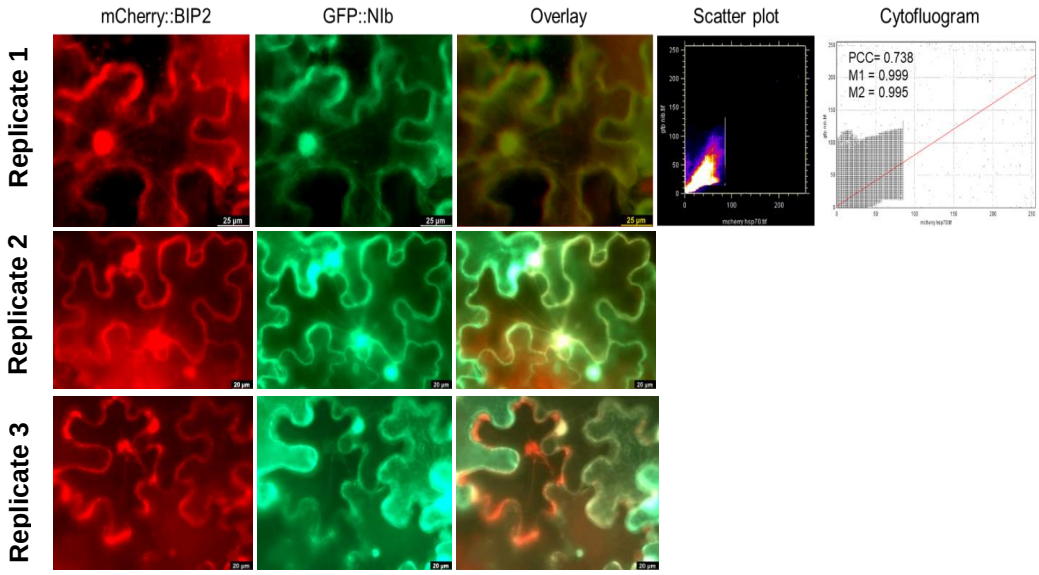

Fig. 7. Sub-cellular localization assay of NbBIP2 and SMV-NIb protein. Both proteins are co-localized in the ER-like compartment (web-like line that extended from nucleus to the cytoplasm). Co-localization analyses were performed by using ImageJ software. A high value of Pearson's correlation coefficient confirms that both of proteins are strongly co-localized in the ER.

V. NbHSP70 homologs in *G. max* were highly regulated by SMV infection.

To analyze homology of genes that encodes HSP70 family members in *G. max,* I constructed phylogenetic tree and discovered several genes in *G. max* located at close distance with genes that encodes HSP70 members in *N. benthamiana* (Fig. 8). To investigate the regulation of these genes, I inoculated *G. max* with SMV strains G5H or G7H. Observation of the GFP expression under UV light was conducted to confirm SMV infection (Fig. 9A). Quantification of relative *GmBIP 1*, *GmBIP 2*, and *GmHSP70-a* expression levels demonstrated that SMV infection regulates the expression of these genes. All of *GmBIP 1*, *GmBIP 2*, and *GmHSP70-a* were upregulated upon infection of SMV strain G5H up to 4 and 12 folds than mock control and up to 2.3, 7, and 6 folds upon infection of SMV strain G7H (Fig. 9B). The upregulation of these genes that encodes HSP70 family member in *G.max* upon SMV infection suggest the importance of these genes in the SMV infection as the one that have been observed in the *N. benthamiana.*

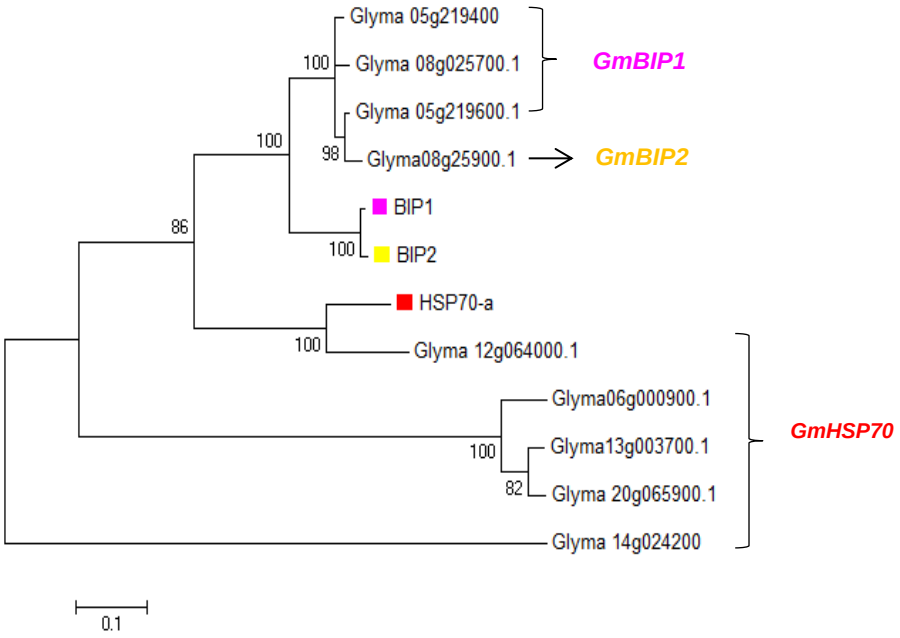

Fig. 8. Phylogenetic analysis of the genes that encode HSP70 family member in *N. benthamiana* and *G. max*. Gene with the annotation *Glyma 12g064000.1* is close related to the *HSP70-a* in *N. benthamiana*. Genes that encodes BIP 1 and BIP 2 of *N.benthamiana* and *G.max* are located at two different clusters, however these clusters are located at the same branch.

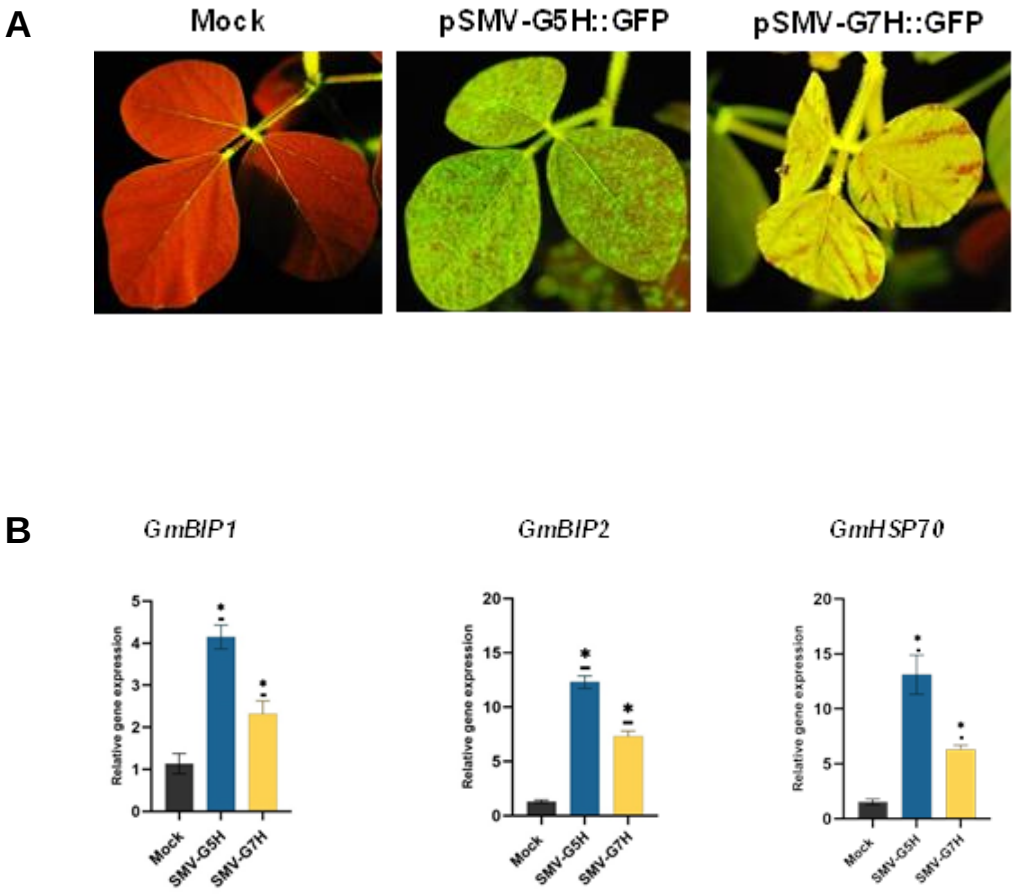

Fig. 9. SMV infection alters the expressing level of genes that encodes HSP70 family member. (A) A GFP expression on *G.max* infected by SMV strain G5H. (B) Expression level of genes that encode HSP70 family member are significantly upregulated compared to the mock control upon the infection of SMV.

DISCUSSION

Management of SMV disease incidence is a crucial step to secure the global soybean production. Up to date, cultivation of resistant soybean cultivars still become the most favorable choice to combat SMV spread in the soybean growing areas. However, this method may not be a good choice for long term approach since SMV was reported enable to modified its genome organization which results in the emerging of various new resistance breaking SMV strains (Ahangaran et al., 2013; Choi et al., 2005; Chowda-Reddy et al., 2011; Gagarinova et al., 2008; Koo et al., 2005). Development of various agricultural techniques in past decade may become an alternative strategy to overcome this problem. One of the most promising methods is by artificially induces new resistance type, known as recessive resistance by manipulating host factors that are necessary for SMV infection (Hashimoto et al., 2016).

A HSP70 is family member that has vital roles in wide range of cellular housekeeping activities including protein folding as well as remodeling processes and also has some roles in response to the biotic and abiotic stresses (Anaraki et al., 2018; Duan et al., 2011; Rosenzweig et al., 2019). HSP70s expressions are induced by many abiotic factors such as heat stress and saline stress, as well as many biotic stresses such as infection of pathogens (Hyskova

et al., 2021). During virus infection, HSP70s can show both positive effects by maintain the plants cellular homeostasis and negative effects as targeted by plant virus to promote their infection (Hyskova et al., 2021). Reports showed that HSP70s were recruited by CP of tomato yellow leaf curl virus and potato virus-A to promote their infection (Gorovits et al., 2013; Hafrén et al., 2010). Increased expression of HSP70s was also observed in response to the infection of several plant viruses including rice stripe virus and CWMV (Jiang et al., 2014; Yang et al., 2017). All of these reports highlight the important of HSP70 proteins in the viral infection that might play dual functions to maintain plants homeostasis and to successful virus infection (proviral). In my study, I observed increased expression of three genes that encodes HSP70 family member, i.e., *BIP1*, *BIP2* and *HSP70-a* in *N. benthamiana* following the infection of SMV (Fig. 2B, 2C, and 2D).

To corroborate the importance of these genes in the SMV infection, I transiently knockdown *BIP 1*, *BIP 2*, and *HSP70*-a in *N.benthamiana* and after confirming the decreased level of each gene (Fig. 3), I challenge inoculated the knockdown lines using SMV strain G5H which expressing GFP signal (Fig. 4). At 14 days post challenge inoculation with SMV, GFP expression was only observed on the wild type *N. benthamiana* infected plants, but not on the knockdown lines (Fig. 4A). Consistently, quantification of the relative SMV

RNA level demonstrated a significant lower level of viral RNA on the knockdown lines compared to wild type infected by SMV (Fig. 4B). Together, these results demonstrated that knockdown of genes which encodes HSP70 family proteins impairs the SMV infection.

Nevertheless, I continued to observe plants condition and possibility of viral movement on the *N. benthamiana* up to 21 days post challenge inoculation by SMV. Interestingly, I observed a terminal death on the *HSP70-a* knockdown plants at the 21 days post challenge inoculation by SMV (Fig. 5A), but not in the *BIP 1* or *BIP 2* knockdown lines. I did not observe any viral accumulation on the upper non-inoculated leaves of both *BIP 1* and *BIP 2* knockdown lines even after 21 dpi. I concluded that HSP70-a is a crucial host factor to maintain plants vitality, and manipulation on this gene causes crucial damage(s) for plants vigor and vitality. On the other hands, BIP proteins that encoded by both of *BIP 1* and *BIP 2* are necessary for SMV infection, evident by inability of SMV to develop infection on the *BIP 1* and *BIP 2* knock-down lines, yet modification one of these genes does not affect plants vitality (Fig. 4 and 5A).

The BIPs are group of proteins from HSP70 family protein that exist in the ER (Buzeli et al., 2002). BIP proteins have been reported to act in multifunctional modulator of the processes that occurs in ER including protein

folding, protein translocation, regulation of the protein secretion and taking part to coordinate the rate of ER protein processing and mRNA translation, and to restore protein folding homeostasis to the ER (Buzeli et al., 2002; Laitusis et al., 1999; Pincus et al., 2010). BIP 1 and BIP 2 are ubiquitously expressed in plant tissues and are identical to each other (Maruyama et al., 2015), this support the idea that BIP 1 and BIP 2 are functionally redundant in maintaining plant's homeostasis, and modification on one of genes that encodes this protein will not affect the plant fitness, yet impairs SMV infection. In my study, the expressions of *BIP 2* are higher compared to *BIP 1* upon SMV infection (Fig. 2B and 2C). Subsequently, I used *BIP 2* gene for further investigation of the importance of HSP70 family member in the SMV infection.

Analyses of the protein structure of *N. benthamiana's* BIP 2 protein demonstrated presence of ER retrieval motif (HDEL motif) (Mei et al., 2017) on the end of C-terminal region (Fig. 6A). Furthermore, subcellular localization assay of BIP 2 on the *N. benthamiana* leaf cells corroborate localization of BIP 2 in the ER, evident by red fluorescence signal line that extended from nucleus to the cytoplasma (Fig. 7). Taking that BIP 2 interacts *in vivo* with SMV replicase, NIb (Fig. 5B), I also analyzed the prediction of protein structure of SMV NIb from both strains G5H and G7H and confirmed the presence of “KKXX” ER retrieval motif on NIb's protein structure (Fig. 6B). H/KDEL and

KKXX motifs are common ER retrieval motifs that play a pivotal role for the localization in the ER (Stornaiuolo et al., 2003). Presence of KKXX motif in the NIb protein structure suggested that this viral protein may localized in the ER. To corroborate this hypothesis I conducted subcellular localization assay and confirmed the localization of NIb protein in the ER of *N.benthamiana*, evident by expression of GFP signals on the ER-like compartment. Subsequently, correlation assay that was performed by PCC and MOC demonstrated that both proteins (BIP 2 and NIb) are co-localized and strongly correlated in the ER (Fig. 7). Taking that, 1) replication of many potyviruses occur in ER; 2) NIb is the core of replication complex in most potyviruses, and 3) most plant viruses including SMV do not possess chaperon to regulate the protein synthesis, folding, and maturation (Nagy et al., 2011), I assumed that SMV-NIb may recruit BIP 2 from the ER into replication complex to promote SMV infection and assist maturation of viral protein.

Finally, I also measured level of homolog genes in *G. max* upon infection of SMV. As expected, expression levels of homolog genes in *G. max* increased upon SMV infection (Fig. 9B), implying that HSP70 family members are essential for SMV infection not only in *N. benthamiana* but also in *G. max*. Therefore, modification of genes that encode HSP70 family member, i.e., *BIP 2*

in many popular soybean cultivars might be an effective alternative method in managing SMV diseases caused especially by resistance breaking SMV strains.

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콩 모자이크 바이러스와 기주 인자 간 상호작용 기작의 구명 : 콩 모자이크 바이러스의 감염에 대한 GmPAP2.1과 HSP 70 단백질의 서로 다른 역할

크리스틴 위드야사리

초록

식물 병원균과 그들의 기주 간의 상호작용은 다양한 기주인자들이 연관되어 있는 복합적인 과정이다. 이러한 상호작용들에 의해 병원체의 성공적인 기주 식물 내의 감염이나 반대로 기주 식물의 저항성 반응이 일어난다. 따라서, 바이러스와 기주인자 간의 상호작용에 관한 연구는 바이러스 감염 과정을 이해하고 나아가 바이러스에 대한 방제를 위한 방법을 개발하는데 더 많은 통찰력을 줄 수 있다. 본 연구에서는, 콩 모자이크 바이러스(SMV)와 상호작용하는 두 가지 콩 기주인자인 GmPAP 2.1 와 BIP2 들의 기능을 구명하고, 이들과 바이러스 간의 서로 다른 기작의 상호작용을 밝히고자 하였다. 우선, purple acid phosphatase, GmPAP 2.1 유전자는 SMV에 저항성을 보이는 cultivar 에서 그 발현이 확인되었고, SMV에 감수성을 보이는 cultivar에

GmPAP 2.1 유전자의 과발현을 유도하면 기주 식물에 저항성을 부여하는 것으로 확인되었다. 이와 같은 저항성 반응은 GmPAP2.1와 SMV의 한 단백질인 P1과의 상호작용에 의한 것으로 보여지며, 이 두 단백질간의 상호작용은 살리실산(SA) 관련 유전자들의 발현량 증가를 야기하는 것을 확인하였다. 살리실산 관련유전자들의 발현의 증가는 SA 의 축적과 SAR 의 활성을 유도하였다. 반면에, *Nicotiana benthamiana* 식물에서의 SMV의 감염은 HSP70 family protein 중의 하나인, BIP2 유전자의 발현량 증가를 야기한다. 해당 유전자를 담배에서 knock-down 할 경우, SMV 의 감염이 일어나지 잘 일어나지 않음을 확인하였다. 세포 내에서의 위치와 Yeast two hybrid 실험을 통해 SMV의 NIb 단백질과 BIP2 단백질이 공통적으로 ER에 존재하며, 이러한 현상은 BIP2 단백질이 SMV 감염을 증진시키기 위해 NIb와의 상호작용을 통하여 viral replication complex를 재구성할 것임을 시사한다. 콩에서도 SMV 감염시 HS70 protein을 암호화하는 유전자의 발현이 증가하는 것으로 보아, 콩-SMV 간의 pathosystem에서도 해당 유전자가 비슷한 역할을 할 것으로 기대된다. 따라서, 본 연구는 SMV 감염 시 서로 반대되는 역할을 하는 기주 인

자들을 새롭게 밝혀 내었으며, 이 두 기주인자들에 관한 연구는 SMV에 대한 콩의 병 저항성 연구와 방제에 관련한 도움이 되는 정보를 제공하게 될 것이다.

Keyword: 콩모자이크바이러스, 콩, 기주인자, 상호작용, 저항성