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requests for materials
should be addressed to
K.H.K. (kookkim@snu.
ac.kr)

Type 2C Protein Phosphatase Is a Key Regulator of Antiviral Extreme Resistance Limiting Virus Spread

Jang-Kyun Seo¹, Sun-Jung Kwon², Won Kyong Cho³, Hong-Soo Choi¹ & Kook-Hyung Kim³

¹Crop Protection Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea, ²Horticultural and Crop Herbal Environment Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 440-310, Republic of Korea, ³Department of Agricultural Biotechnology and Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-921, Republic of Korea.

Effector-triggered immunity (ETI) is an active immune response triggered by interactions between host resistance proteins and their cognate effectors. Although ETI is often associated with the hypersensitive response (HR), various R genes mediate an HR-independent process known as extreme resistance (ER). In the soybean-*Soybean mosaic virus* (SMV) pathosystem, the strain-specific CI protein of SMV functions as an effector of *Rsv3*-mediated ER. In this study, we used the soybean (*Rsv3*)-SMV (CI) pathosystem to gain insight into the molecular signaling pathway involved in ER. We used genome-wide transcriptome analysis to identify a subset of the type 2C protein phosphatase (*PP2C*) genes that are specifically up-regulated in *Rsv3*-mediated ER. Gain-of-function analysis of the most significantly expressed soybean *PP2C* gene, *GmPP2C3a*, showed that ABA-induced *GmPP2C3a* functions as a key regulator of *Rsv3*-mediated ER. Our results further suggest that the primary mechanism of ER against viruses is the inhibition of viral cell-to-cell movement by callose deposition in an ABA signaling-dependent manner.

Plants fight pathogen attack via a complex immune system that includes basal and highly specific defense responses¹⁻³. The first line of defense against the vast majority of potential pathogens is termed pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI)¹⁻². To circumvent PTI, many host-adapted pathogens produce different classes of effector molecules¹⁻². In turn, plants have evolved a second surveillance mode to recognize pathogen-derived effector proteins. This second mode or line of defense is triggered by specialized molecules such as resistance (R) proteins, which recognize the effectors and activate effector-triggered immunity (ETI)¹⁻². When R genes recognize effector proteins, the typical resistance responses include the hypersensitive response (HR) and extreme resistance (ER)⁴⁻⁵. HR results in localization of the pathogen at the inoculation site as a consequence of the rapid death of infected and neighboring cells⁵. In contrast, ER is characterized by the arrest of pathogens at the inoculation site without any macroscopic or microscopic cell death lesions^{4,6}.

Soybean mosaic virus (SMV), a member of the genus *Potyvirus*, is one of the most common viruses of soybean (*Glycine max* (L) Merr.)⁷. SMV has a single-stranded positive-sense RNA genome of approximately 9.6 kb. This genome encodes one large polyprotein, which is proteolytically cleaved to generate at least 11 mature proteins: P1, helper-component proteinase (HC-Pro), P3, PIPO, 6K1, cylindrical inclusion (CI), 6K2, VPg, NIa-Pro, NIB, and coat protein (CP)⁸. SMV has been classified into several distinct strains based on disease reactions on various soybean genotypes⁹⁻¹⁰. Genetic mapping studies have identified three distinct R genes (*Rsv1*, *Rsv3*, and *Rsv4*) that confer strain-specific resistance to SMV¹¹⁻¹⁴. Extensive studies have identified the SMV effector proteins that interact with each *Rsv* gene: SMV strain-specific P3 protein is an effector of both *Rsv1*- and *Rsv4*-mediated disease resistance¹⁵⁻¹⁶ while SMV CI protein is an effector of *Rsv3*-mediated ER¹⁷⁻¹⁸.

The plant hormone abscisic acid (ABA) not only regulates various plant developmental processes but also has a primary function in plant response to biotic and abiotic stress¹⁹⁻²⁰. Accumulating evidence suggests that ABA has many effects on defense responses, acting as both a positive and negative modulator of associated signaling pathways; its effects depend on the specific plant-pathogen interaction¹⁹. The key regulators of the ABA signaling network are a subclass of serine/threonine phosphatases, represented by type 2C protein phosphatases (*PP2Cs*)²¹⁻²². The large number of *PP2C* genes in plant genomes suggests that the integrative network of ABA signaling is finely orchestrated by the diversity and specificity of *PP2Cs*²².



Although *Rsv3*-mediated ER induced in response to SMV attack was phenotypically characterized in the previous studies^{17–18}, the involved molecular mechanism remains unclear. In the current study, we gained insight into the molecular mechanism involved in *Rsv3*-mediated ER by using RNA sequencing (RNA-Seq) to analyze the genome-wide transcriptional reprogramming that occurs in soybean expressing ER to SMV. In *Rsv3*-mediated ER, we observed a significant up-regulation of a subset of the *PP2C* genes that are induced by ABA. We focused on the *PP2C* gene that was most up-regulated, which was *GmPP2C3a*. Using infectious clones of avirulent and virulent strains of SMV (SMV-G5H and -G7H, respectively) tagged with a β -glucuronidase (GUS) reporter gene, we show that *Rsv3*-mediated ER restricts virus accumulation in the initially inoculated cells. Synchronized over-expression of *GmPP2C3a* in the cell inoculated with SMV-G7H results in ER, suggesting that *GmPP2C3a* is a key regulatory gene conferring ER. Further evidence suggests that inhibition of viral cell-to-cell movement by *Rsv3*-mediated ER is primarily caused by callose deposition in an ABA signaling-dependent manner.

Results

The strategy used here for comparative analysis of genome-wide transcriptional regulation of *Rsv3*-mediated ER is outlined in Figure 1. Infectious sap of SMV-G5H (an avirulent strain) was mechanically inoculated on the fully expanded first trifoliolate leaves of the soybean line L29 carrying *Rsv3*. For controls, the soybean leaves were inoculated with mock and SMV-G7H (a virulent strain) in the same way. For time-course analysis, mRNAs, which were extracted from the inoculated leaves and untreated healthy leaves at 8, 24, and 54 hours post inoculation (hpi), were subjected to RNA-Seq. To identify genes specifically involved in *Rsv3*-mediated ER, we evalu-

ated differentially expressed genes among the samples, i.e., the expression levels of the genes were calculated relative to those of the corresponding genes in the untreated, healthy control (see Methods).

Specific up-regulation of a subset of the *PP2C* gene family in *Rsv3*-mediated ER. We performed extensive analysis of the RNA-Seq data for gene families categorized by biological function (according to the SoyBase database). Using this approach, we found a significant up-regulation of a subset of the *PP2C* gene family in *Rsv3*-mediated ER at an early time point (8 hpi) (Figs. 2 and S1). Phylogenetic analysis of the soybean *PP2C* gene family revealed that the up-regulated *PP2C* genes belonged to the same cluster that contains the soybean orthologs of *Arabidopsis ABI1* and *ABI2*, which are negative regulators of ABA responses^{23–24} (Fig. S1B). However, the expression levels of the soybean orthologs of *ABI1* and *ABI2* were not significantly different from those of the controls, indicating that the regulatory mechanism of *Rsv3*-mediated ER differs, at least in the identity of the ABA-inducible genes, soybean vs. *Arabidopsis* (Fig. S1). The expression levels of the significantly up-regulated *PP2C* genes were quantified as reads per kilobase of the transcript per million mapped reads to the transcriptome (RPKM). Among them, Glyma14g32410, which is referred to as *GmPP2C3a* in this study, was the most highly expressed in *Rsv3*-mediated ER at an early time point (8 hpi) (Fig. 2C). The high *GmPP2C3a* expression level indicated by RNA-Seq was confirmed by real-time PCR (Fig. 2D). Thus, we subjected *GmPP2C3a* to detailed analysis with the goal of determining its functional involvement in *Rsv3*-mediated ER.

ABA accumulation accompanies *Rsv3*-mediated ER. Because some *Arabidopsis PP2C* genes including *ABI1* and *ABI2* are

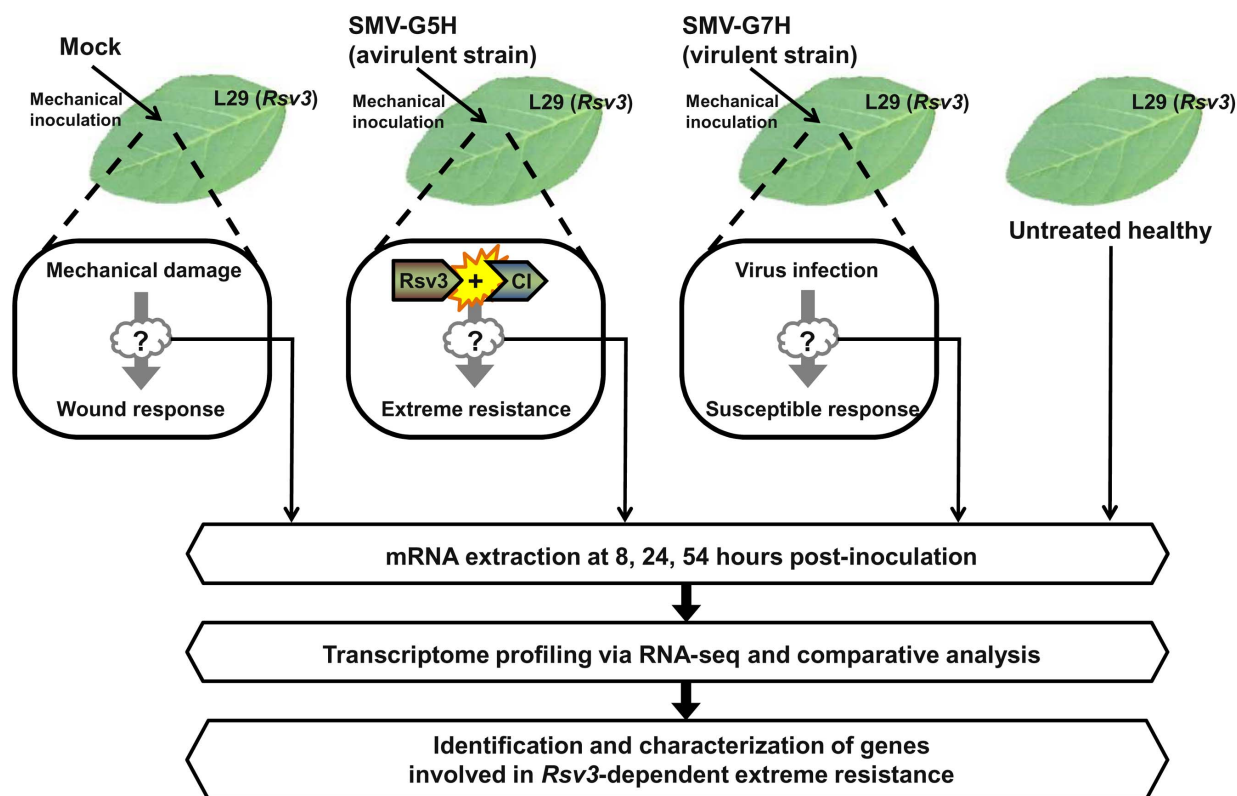


Figure 1 | The strategy for comparative analysis of genome-wide transcriptional regulation of *Rsv3*-mediated extreme resistance. The fully expanded first trifoliolate leaves of the soybean line L29 carrying *Rsv3* were mechanically inoculated with either mock, SMV-G5H (avirulent strain), or SMV-G7H (virulent strain). For time-course analysis, mRNAs were extracted from the inoculated leaves and untreated healthy leaves at 8, 24, and 54 hours post inoculation (hpi) and subjected to RNA-Seq. To identify genes specifically involved in *Rsv3*-mediated ER, comparative analyses of the transcriptome profiles were performed. The image of the leaf in Fig. 1 is a photograph taken by one of our co-authors.

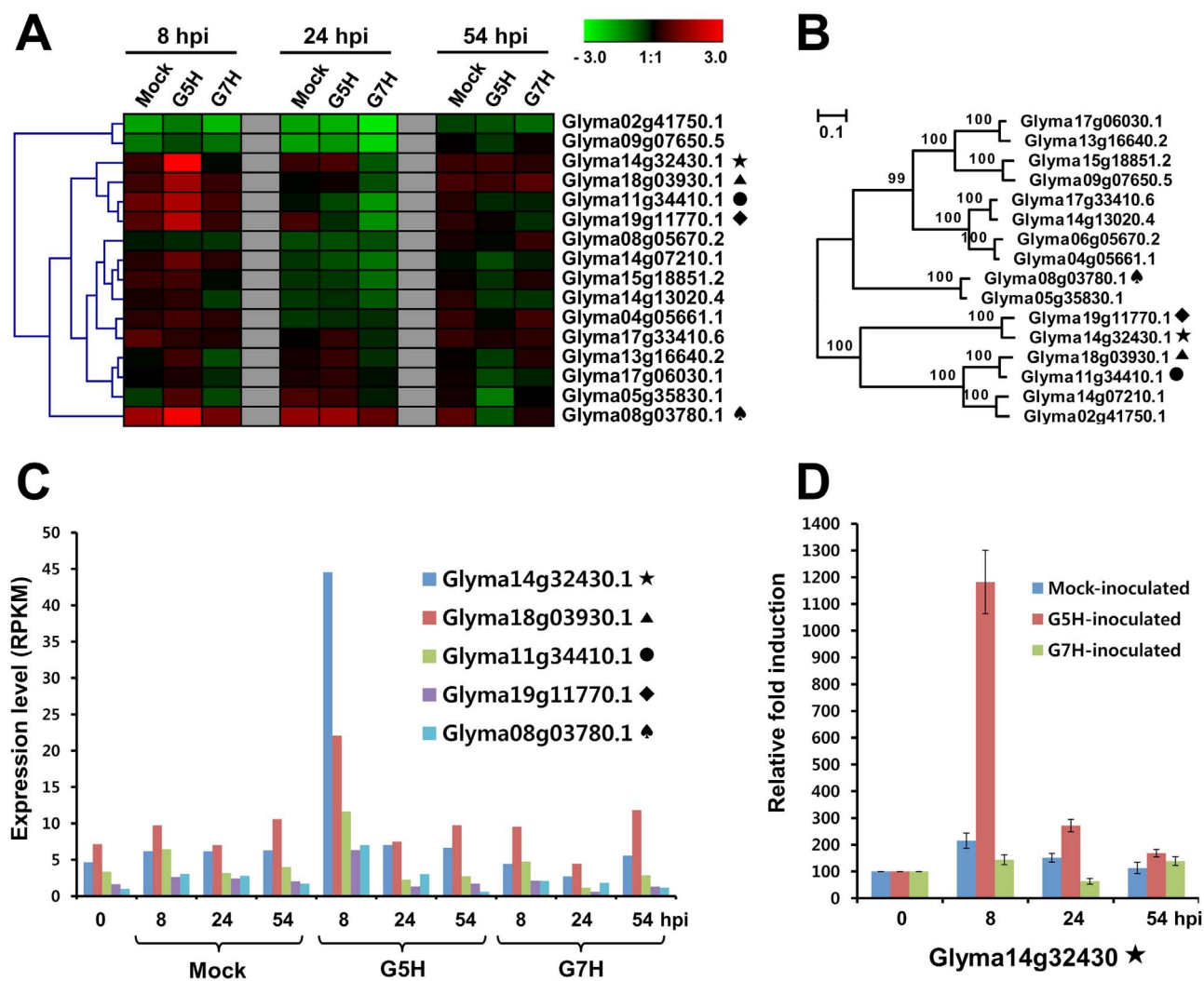


Figure 2 | Expression levels of the *PP2C* genes inducible by ABA in *Rsv3*-mediated ER. **(A)** The heat map showing the hierarchically clustered expression patterns of 16 soybean *PP2C* genes inducible by ABA. The heat map was produced using a log scale of the values of RPKM (reads per kilobase of the transcript per million mapped reads to the transcriptome) obtained by RNA-Seq. Red and green indicate up-regulation and down-regulation, respectively. **(B)** Phylogenetic analysis of 16 soybean *PP2C* genes inducible by ABA. The analysis was performed using the deduced amino acid sequences by the maximum likelihood method. **(C)** The RPKM values of five significantly up-regulated *PP2C* genes. **(D)** Real-time PCR validation of the expression level of Glyma14g32410, which is referred to as *GmPP2C3a* in this study. Symbols indicate the significantly up-regulated *PP2C* genes in *Rsv3*-mediated ER at 8 hpi.

transcriptionally up-regulated by ABA^{25–26}, the endogenous levels of ABA were analyzed in soybean leaves inoculated with either SMV-G5H or SMV-G7H. As expected, ABA accumulation was significantly higher in the leaves inoculated with SMV-G5H than in those inoculated with mock or SMV-G7H (Fig. 3A). Because ABA has an antagonistic effect on SA signaling that plays an important role in activation of defense responses, the accumulation levels of SA were also analyzed in the same set of the soybean leaf samples. Interestingly, SA accumulation was dramatically increased in the SMV-G7H-inoculated leaves while no significant difference in SA accumulation was observed between SMV-G5H- and mock-inoculated leaves (Fig. 3B). Taken together, the results suggested that the ABA signaling pathway might be involved in conferring *Rsv3*-mediated ER and that *GmPP2C3a* could function as a key regulator of this signaling pathway. In addition, it seemed likely that although the SA signaling pathway could be activated by SMV infection, the activation was insufficient to confer resistance to virulent strains of SMV.

Synchronized overexpression of *GmPP2C3a* inhibits virus cell-to-cell movement. We previously developed a useful viral vector system

by engineering SMV-G7H to express foreign genes in soybean and by tagging the SMV vector with a GUS gene; using this system, we were able to visualize virus infection and movement in plant cells^{27–28}. The same strategy was used to tag SMV-G5H with GUS (Fig. 4A). We hypothesized that if *GmPP2C3a* is a positive regulator of *Rsv3*-mediated ER, its ectopic overexpression would confer ER even to a virulent strain of SMV. Testing this hypothesis required that *GmPP2C3a* be overexpressed only in the cells infected with SMV. Thus, we used the SMV-G7H vector for the synchronized overexpression of *GmPP2C3a*, and we named the resulting construct that was engineered to overexpress *GmPP2C3a* as pSMV-G7H-*GmPP2C3a* (Fig. 4).

We first examined the infectivity of pSMV-G7H-*GmPP2C3a*. Plasmid DNAs of pSMV-G7H-*GmPP2C3a* and other controls were rub-inoculated onto the first trifoliolate leaves of soybean line L29 (*Rsv3*). While all of the soybean plants inoculated with pSMV-G7H and pSMV-G7H-GUS were systemically infected (Table 1), no infection of the soybean plants inoculated with pSMV-G5H or pSMV-G7H-*GmPP2C3a* was evident even at 25 days post inoculation (dpi), as determined by subjecting the total RNA extracted from upper non-inoculated leaves to RT-PCR specific for SMV (Table 1). The

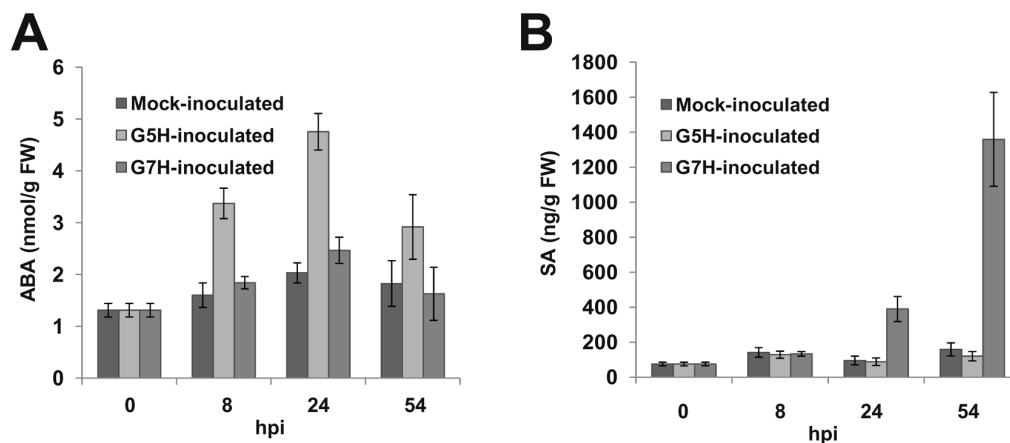


Figure 3 | Time-course analysis of ABA and SA levels in *Rsv3*-mediated ER. Endogenous ABA (A) and SA (B) levels in soybean leaves inoculated with mock, SMV-G5H, or SMV-G7H were quantified at 8, 24, and 54 hpi. Values are the means \pm SD from three independent experiments.

results suggested that the synchronized overexpression of GmPP2C3 might induce resistance to SMV-G7H even in the absence of *Rsv3*-dependent activation. However, we could not totally exclude the possibility that the presence of GmPP2C3a DNA sequence in the viral vector genome is responsible for the impaired infectivity, although foreign gene expression by proteolytic processing from the SMV polyprotein precursor is a promising strategy. Therefore, we performed additional experiments as described below.

The most striking feature of ER is the rapid arrest in an HR-independent manner of virus accumulation in the initially infected cell⁴. Thus, we next examined the phenotypic characteristics of *Rsv3*-mediated ER on virus accumulation at the microscopic level. To this end, we inoculated L29 (*Rsv3*) with pSMV-G5H-GUS and pSMV-G7H-GUS and performed a histochemical GUS assay at 5 dpi to visualize virus accumulation and cell-to-cell movement because only infected cells express GUS activity as a reporter of virus accumulation²⁸. SMV-G7H-GUS replicated actively and spread to form infection foci > 300 μ m in diameter (Fig. 5A and Table S1). In contrast, accumulation of SMV-G5H-GUS was restricted to the initially inoculated single cells (Fig. 5A and Table S1). However, *Rsv3*-mediated ER did not seem to inhibit virus replication in the initially infected cells because high levels of GUS activity produced by replication of SMV-G5H-GUS were detectable (Fig. 5A).

We next determined whether the synchronized overexpression of GmPP2Ca results in restriction of virus accumulation in the initially infected cells as observed above. Thus, we engineered the *GmPP2C3a* coding sequence into the polyprotein ORF between the N1b and CP cistrons of pSMV-G7H-GUS to produce mature GmPP2C3a by proteolysis (Fig. 4). The resulting construct, named pSMV-G7H-GUS-GmPP2C3a, could produce both GUS and GmPP2C3a upon virus replication. Leaves of L29 (*Rsv3*) were inoculated with pSMV-G7H-GUS-GmPP2C3a and subjected to a GUS assay. As expected, virus accumulation was restricted to the initially inoculated single cell and obvious detection of the GUS expression confirmed that the insertion of GmPP2C3a in the viral genome did not impair virus replicative ability (Fig. 5A and Table S1). This result suggested that ectopic overexpression of GmPP2C3a could activate ER in the absence of effector recognition by *Rsv3*.

Callose deposition is critical for *Rsv3*-mediated ER. Cell-to-cell movement of a plant virus through plasmodesmata (PD) is a prerequisite for systemic infection^{29–30}. Callose deposition is known to decrease the permeability of PD and therefore virus movement through PD^{31–33}. Thus, we determined whether *Rsv3*-mediated ER is accompanied by callose deposition at PD. Leaves of L29 (*Rsv3*) that had been inoculated with pSMV-G7H-GUS, pSMV-G5H-GUS, or pSMV-G7H-GUS-GmPP2C3a were stained with aniline blue and

then examined microscopically at 5 dpi. Interestingly, significant quantities of callose accumulated along the plasma membrane of the initially inoculated single cells in the leaves inoculated with pSMV-G5H-GUS and pSMV-G7H-GUS-GmPP2C3a but not in leaves inoculated with mock or pSMV-G7H-GUS (Fig. 5B).

To further confirm whether the callose deposition detected by microscopic observation was relevant for *Rsv3*-mediated ER, we performed inoculation experiments in L29 (*Rsv3*) and included treatments of 500 μ M 2-deoxy-D-Glc (DDG), which is a callose synthesis inhibitor³⁴. DDG was applied two-times: first at 24 hours before inoculation and then at 24 hours after inoculation. Interestingly, inoculation of pSMV-G5H and pSMV-G7H-GmPP2C3a resulted in HR-like necrotic lesions on the leaves treated with DDG but not on those treated with H₂O, while no visible lesions were detected on the leaves inoculated with pSMV-G7H and other controls (Fig. 5C). In addition, a significant accumulation of viral RNA was demonstrated by RT-PCR of leaf tissues with HR-like necrotic lesions (data not shown). The results indicated that inhibition of callose deposition resulted in the failure to arrest viruses at the initially inoculated sites even though *Rsv3*-mediated defense signaling was activated. Furthermore, our results suggest that *Rsv3*-mediated signaling has the potential to induce a cell death response although it seems that ER is epistatic to the cell death response in *Rsv3*-mediated resistance. This further suggested that GmPP2C3a induction could be associated with both ER and HR as a general feature of ETI in soybean.

Discussion

ETI is a rapid and strong immune response activated by interactions between host R proteins and their cognate effectors^{1–2}. Although HR, a form of programmed cell death, is a common manifestation of ETI, various R genes mediate HR-independent processes that confer resistance against viruses; these HR-independent responses are referred to as extreme resistance or ER^{4,17,35}. While the HR signaling pathway

Table 1 | Effect of the synchronized expression of GmPP2C3a on SMV infectivity in three independent experiments

Inoculum*	Exp. 1	Exp. 2	Exp. 3	Total
Mock	0/3 [†]	0/3	0/3	0/9
pSMV-G7H	5/5	5/5	5/5	15/15
pSMV-G5H	0/5	0/5	0/5	0/15
pSMV-G7H-GUS	5/5	5/5	5/5	15/15
pSMV-G7H-GmPP2C3a	0/5	0/5	0/5	0/15

*Soybean line L29 (*Rsv3*) plants were inoculated with the corresponding plasmids.

[†]Number of systemically infected plants/number of plants inoculated. Virus infection of the upper non-inoculated leaves was confirmed by RT-PCR using SMV-specific primers at 21 dpi.

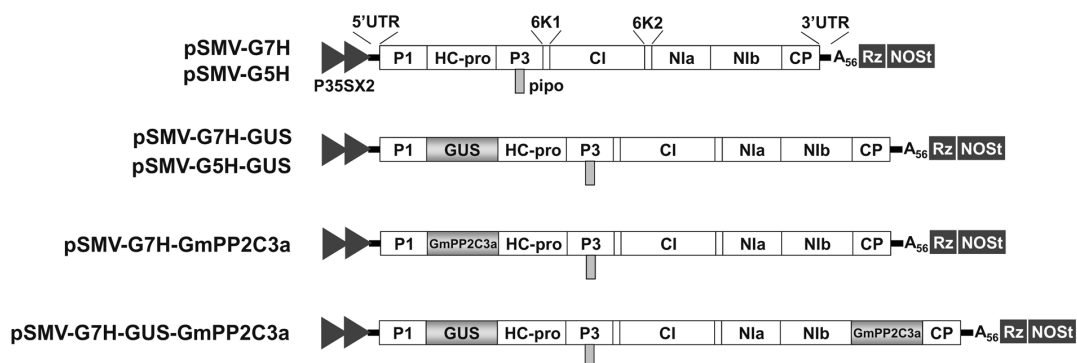


Figure 4 | Schematic representation of the SMV infectious constructs. The gene of interest was inserted between either P1 and HC-Pro or between NIB and CP to be generated by proteolysis when the virus replicates. *In vivo* transcription of the SMV infectious constructs is under the control of a double 35S promoter (P35SX2), a cis-cleaving ribozyme sequence (Rz), and a NOS terminator (NOST).

has been extensively studied in various host-pathogen systems^{1–2}, very limited information is available on the signaling pathway involved in ER.

The soybean-SMV pathosystem is a useful model for studying molecular interactions between host and viruses, because the genotype-specific disease responses are well characterized for various SMV strains^{17,35–37}. In this study, we used the soybean (*Rsv3*)-SMV (*CI*) pathosystem to gain insight into the molecular signaling pathway involved in ER. Genome-wide transcriptome analysis enabled the identification of a subset of *PP2C* genes that are significantly

induced in *Rsv3*-mediated ER. The most significantly expressed soybean *PP2C* gene, *GmPP2C3a*, was selected for in depth study to further characterize its functional involvement in *Rsv3*-mediated ER.

A major mechanism of signal transduction is reversible phosphorylation of protein components in the particular pathway²¹. *PP2Cs* form the largest protein phosphatase family in plants, a family that is also highly complex. In eukaryotes, *PP2Cs* act as negative regulators of stress-activated protein kinase cascades^{21–22}. In plants, substantial research has shown that *PP2Cs* function as negative regulators of ABA signaling^{21–22}. *ABI1* and *ABI2* are the best-characterized plant

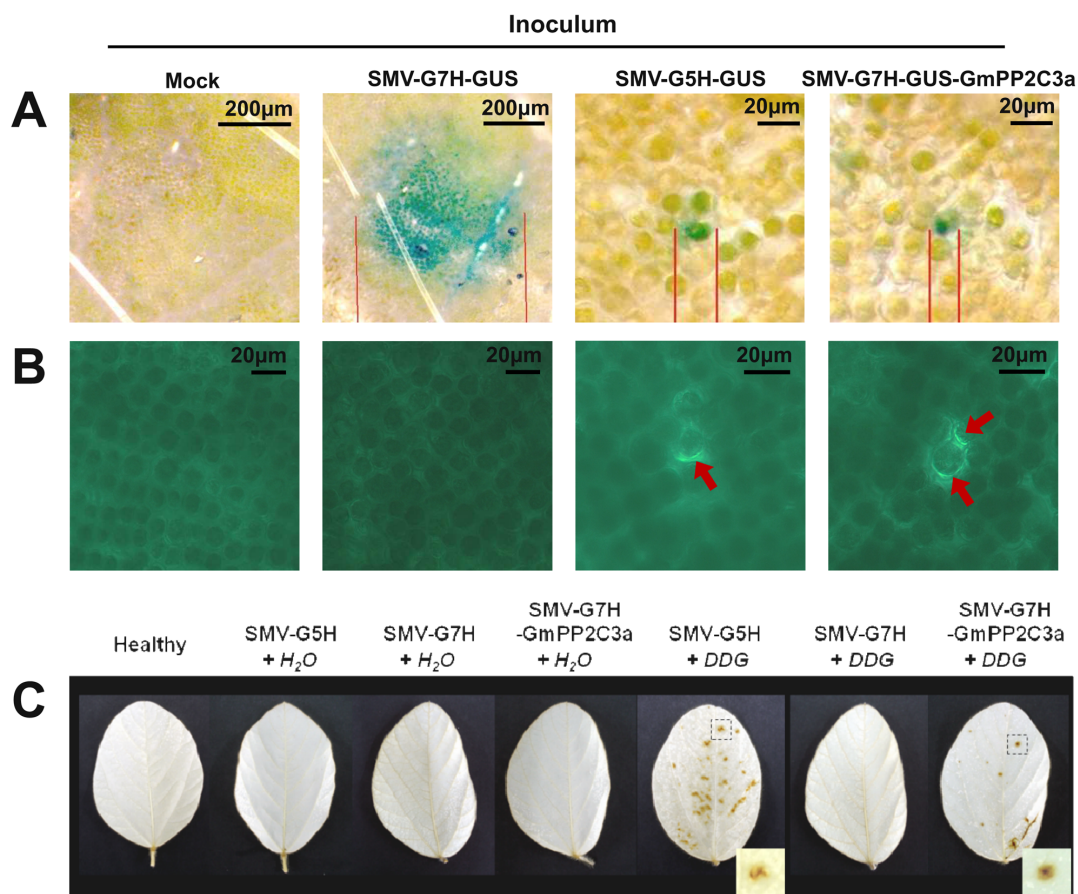


Figure 5 | *GmPP2C3a* restricts virus accumulation and enhances callose deposition in *Rsv3*-mediated ER. *In situ* detection of GUS activity (A) and callose deposition (B) in soybean leaves. The inocula are indicated above each image. Red arrows indicate accumulation of callose stained with aniline blue. The GUS foci and callose deposition were photographed at 5 dpi. (C) The appearance of HR-like necrotic lesions on the leaves inoculated with SMV-G5H and SMV-G7H-*GmPP2C3a* when treated with DDG. The enlargements of the boxed areas are shown below each image. Images were captured at 8 dpi.



PP2Cs and are partially redundant in their repression of ABA responses^{24–25}. ABI1 and ABI2 function as key regulators of ABA signaling by being responsible for nearly 50% of the ABA-induced PP2C activity²⁴. However, this also indicates that other PP2Cs are involved in the regulation of the integrative network of ABA signaling. Indeed, accumulating evidence shows that PP2Cs are involved in regulation of plant immunity as downstream factors of ABA signaling^{38–40}. In this study, we identified a subset of the PP2C gene family that is significantly up-regulated soon after inoculation with the SMV avirulent strain G5H (Fig. 2 and S1). However, this PP2C gene subset did not include the soybean orthologs of ABI1 and ABI2 (PP2Cs that are up-regulated by ABA in *Arabidopsis*), although all of these PP2Cs are closely related according to phylogenetic analysis (Fig. S1). Our results suggest that the up-regulated PP2C gene subset may be specifically involved in Rsv3-mediated ER in an ABA-dependent manner. This further suggests that the PP2C activity is regulated by protein expression level and that PP2Cs finely modulate certain signal pathways of ABA signaling through their diversity and specificity to substrates and other signaling components.

Rsv3-mediated ER was accompanied by ABA accumulation (Fig. 3A). In *Arabidopsis*, some of the PP2Cs, including ABI1, ABI2, and AtPP2CHA, are transcriptionally up-regulated by ABA²¹. In Rsv3-mediated ER, however, and as noted earlier, elevated ABA triggered up-regulation of a certain subset of soybean PP2Cs including GmPP2C3a but not the orthologs of ABI1 and ABI2 (Fig. 2 and S1). Because the effect of ABA on plant defense was shown to be ambivalent, researchers have proposed various modes of action of ABA on disease resistance, including antagonistic suppression of SA- and ET/JA-mediated basal defense, synergistic stimulation of JA signaling, induction of stomatal closure, and priming for callose deposition^{19–20,41}. Thus, it is likely that the complex role of ABA in defense responses is modulated by divergent signaling pathways and that phase-specific activation of certain PP2Cs determines the modes of action of ABA-dependent disease responses.

As mentioned above, one of the positive effects of ABA on plant immunity is its ability to trigger callose deposition³². Callose can serve as a matrix in which defense molecules are deposited, and its deposition and degradation at the neck region of plasmodesmata (Pd) is one of the regulatory mechanisms controlling Pd permeability^{32–33}. Successful infection of host plants by viruses requires cell-to-cell movement through Pd and long-distance movement through the vascular tissue⁴². In this regard, callose deposition at PD can serve as an antiviral defense response by forming a physical barrier, thereby preventing virus cell-to-cell movement and restricting virus accumulation at the initially infected cells³³. Our results showed that Rsv3-mediated ER is associated with callose deposition and that ectopic overexpression of GmPP2C3a could activate callose deposition (Fig. 5), indicating that GmPP2C3a is a positive regulator of callose deposition. Accumulation of callose is primarily controlled by the balance of activities of callose synthases and the β -1,3-glucanases that degrade callose³³. Thus, GmPP2C3a may function upstream of callose synthases and/or β -1,3-glucanases in regulation of callose accumulation.

Based on our findings, we suggest a hypothetical model for Rsv3-mediated ER against SMV (Fig. 6). In the initially infected cells, SMV replicates and produces the effector CI. The recognition of CI by the R protein Rsv3 results in accumulation of ABA and thus activation of the ABA signaling pathway. In the ABA signaling pathway linked to Rsv3-mediated ER, the transcriptionally up-regulated PP2C genes, including GmPP2C3a, function as positive regulators of the signaling to stimulate callose deposition. Eventually, callose deposition at PD inhibits virus cell-to-cell movement and restricts virus accumulation to the initially infected cells.

ETI is the outcome of dynamic interactions between plants and pathogens^{1–3}. Various factors including interaction affinity between an R protein and its effector, levels of interacting components, and

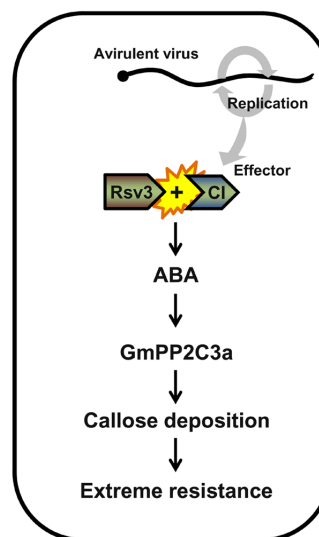


Figure 6 | A model for Rsv3-mediated ER against SMV.

timing may affect the fine tuning of the defense signaling network^{1–3}. Thus, it is important to find and characterize key regulators that decide the fate of resistance interactions. Based on genome-wide transcriptome analysis, we have identified a soybean PP2C gene, GmPP2C3a, that is a key regulator of Rsv3-mediated ER. To further dissect Rsv3-mediated ER, researchers will need to isolate GmPP2C3a-interacting proteins and signaling components that link GmPP2C3a to callose deposition.

Methods

Plants, virus sources, and inoculation. Soybean line L29 (Rsv3) was grown in a growth chamber at 25°C under a 16/8-h photoperiod. Soybean seedlings were selected for inoculation when the first trifoliolate leaves were fully expanded. Infectious cDNA clones of SMV-G7H (pSMV-G7H)²⁸ and SMV-G5H (pSMV-G5H)⁴³ were used as the virus sources. SMV G7H and G5H strains were propagated in the soybean cultivar Lee 74 (rsv) and were used for sap inoculation. The saps and cDNA plasmids of infectious SMV constructs were mechanically inoculated as described previously²⁸. Where indicated, soybean leaves were treated with either 500 μ M DDG or H₂O two times: at 24 hours before inoculation and at 24 hours after inoculation.

Transcriptome analysis. Inoculation experiments described in Figure 1 were carried out independently three times, with each treatment being represented by at least 10 plants in total. In this study, one of the primary goals of transcriptome analysis was to identify candidate genes involved in the plant immune responses against viral infection. Although it is important to use biological replication to obtain robust conclusions about biological differences among samples, the cost of RNA-Seq is high, and our analysis was therefore limited to a single biological sample of pooled tissues. Total RNA was extracted from the inoculated leaves and pooled in equal quantities for each experimental set. RNA libraries were generated using the Illumina TruSeq RNA sample prep kit (Illumina, Inc., USA) with no modifications to the standard protocol. Sequencing on an Illumina HiSeq2000 sequencer (Illumina, Inc., USA) was performed by Macrogen Inc. (Seoul, South Korea). Raw sequence reads were filtered by the Illumina pipeline and mapped to reference sequences (Phytozome v9.1, <http://phytozome.net/>) of the soybean genome (*Glycine max*). To identify differentially expressed genes and obtain expression values, statistical comparison between the libraries was performed by calculating RPKM after normalization using the NGS Cell V 4.06 program with default parameters (CLC Bio, Denmark). Heat maps were generated and visualized using Genesis⁴⁴. To validate RNA-seq data, real-time PCR was performed using ACT11 as a reference gene as described previously⁴⁵.

Measurement of ABA and SA Levels. Endogenous ABA and SA were extracted from soybean leaves and then quantified with the Phytodetek ABA Test Kit (Agdia, USA) for ABA and with reversed-phase HPLC (Waters, USA) for SA as described previously⁴⁰.

Engineering of the SMV viral constructs. The coding region of the GmPP2C3a gene was amplified by PCR using a primer pair harboring XbaI sites (5'-TGCTCTAG-AATGTTTCAGTTCGTTGCATGCAAAA-3' and 5'-TGCTCTAGAATCAG-TTGTACTGATCCTCTTAG-3'). The amplified fragments were digested with XbaI and cloned into pSMV-MCS⁴⁶, which was opened with XbaI. The resulting construct with the GmPP2C3a insert in the correct orientation was named pSMV-



G7H-GmPP2C3a. The same strategy was used to construct SMV-G5H-based viral vector carrying the cloning sites (*Xba*I and *Mlu*I) between P1 and HC-Pro as described previously²⁸, and the amplified *GUS* gene was inserted into the *Xba*I site to generate pSMV-G5H-GUS as described previously²⁷. For simultaneous expression of *GUS* and GmPP2C3a, an additional *Nla*-Pro cleavage site and the cloning sites (*Sal*I and *Sna*BI) were engineered into the polyprotein ORF between the Nib and CP cistrons of pSMV-G7H-GUS as described previously²⁷; the resulting construct was named pSMV-G7H-GUS-MCS. The amplified *GmPP2C* fragment was inframe inserted into the *Sal*I site of pSMV-G7H-GUS-MCS, and the resulting construct, which is capable of simultaneously expressing *GUS* and GmPP2C3a, was named pSMV-G7H-GUS-GmPP2C3a.

Detection of GUS activity and callose deposition. GUS expression driven by virus infection was examined at 5 dpi by histochemical GUS assays as described previously²⁷. Detection of callose deposition was performed at 5 dpi by the aniline blue staining method as described previously⁴⁸.

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Author contributions

J.-K.S., S.-J.K. and K.-H.K. designed the experiments. J.-K.S. and S.-J.K. performed the experiments. J.-K.S., S.-J.K., W.-K.C., H.-S.C. and K.-H.K. analyzed the data. J.-K.S., S.-J.K. and K.-H.K. wrote the manuscript. All authors have read and approved the manuscript.



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