Polysaccharide Peptide–Induced Virus Resistance Depends on Ca^{2+} Influx by Increasing the Salicylic Acid Content and Upregulating the Leucine-Rich Repeat Gene in *Arabidopsis thaliana*

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Plant viral diseases cause severe economic losses in agricultural production. The development of biosource-derived antiviral agents provides an alternative strategy to efficiently control plant viral diseases. We previously reported that the exogenous application of polysaccharide peptide (PSP) exerts significant inhibitive effects on *Tobacco mosaic virus* infection in *Nicotiana tabacum*. In this study, we studied in additional detail the mechanism by which PSP can induce virus resistance in *Arabidopsis thaliana*. We found that PSP significantly induced Ca^{2+} influx and increased the accumulation of hydrogen peroxide and salicylic acid (SA) in the *A. thaliana* cells. A gene with a toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat domain (*LRR*) was obtained by RNA sequencing in combination with the screening of the gene-deletion mutants of *A. thaliana*. The *LRR* gene was deleted, and the inductive response of *A. thaliana* to PSP was significantly attenuated after mutation. After the heterologous overexpression of the *LRR* gene in *N. benthamiana*, the SA content and *PR1* gene expression in *N. benthamiana* were significantly increased. Through analyses of the *LRR* gene expression and the ability of *A. thaliana* to resist *Cucumber mosaic virus* following the treatments of PSP and PSP +

ethyleneglycol-bis (beta-aminoethylether)-*N,N'*-tetraacetic acid, it was shown that PSP enhanced the virus resistance of *A. thaliana* by inducing Ca^{2+} influx and subsequently improving expression of the *LRR* gene, which further increased the SA content.

Plant viral diseases have long been a threat to the sustainable development of agricultural production (Mandadi and Scholthof 2013; Scholthof et al. 2011). The global annual economic loss of agriculture caused by plant virus diseases is approximately \$60 billion, with \$20 billion due to losses in field crops (Xie et al. 2009). Unlike fungi and bacteria, plant viruses are parasitic to the host and plants lack a complete immune system against viruses. Therefore, when plants are infected, viral diseases are difficult to control (Mandadi and Scholthof 2013). A considerable amount of research has been conducted over the past several decades to develop strategies to efficiently control viral diseases. Traditional methods to control viral diseases include genetic improvements to breed virus-resistant cultivars by classical crossing (Solomon-Blackburn and Barker 2001; Wang et al. 2011), genetic transformation (Prins et al. 2008), and the use of virus-free plants (Laimer and Barba 2011). The development of antiplant virus agents provides an alternative way to control viral diseases efficiently (Li et al. 2007; Zhao et al. 2017).

Some biogenic polysaccharides, such as chitin (Cao et al. 2014; Hayafune et al. 2014; Liao et al. 2017; Wan et al. 2012), lentinan (Wang et al. 2013), and seaweed polysaccharide (Jiao et al. 2011; Morya et al. 2012; Pardee et al. 2004), induce plant resistance to viral diseases through the regulation of the immune system to the virus. Wang et al. (2009a) found that burdock polysaccharides increase antiviral activity in *Nicotiana tabacum* plants infected with *Tobacco mosaic virus* (TMV) by activating the expression of several defense genes. Kobayashi et al. (1987) and Wang et al. (2013) reported improved virus resistance in lentinan-treated *N. tabacum* plants infected with TMV by inducing the transient production of hydrogen peroxide (H_2O_2) and the expression of some defense-related genes. Pardee et al. (2004) also reported such improved resistance in algal polysaccharide-treated *Chenopodium quinoa* plants infected with *Potato virus X* by aggregation of the virus particles. Shibuya and Minami (2001) enhanced the antiviral property in oligosaccharide-treated *N. tabacum* plants infected with TMV by improving the

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defense responses of plants. Oligosaccharides enhance plant disease resistance by increasing the nitric oxide and H₂O₂ levels and Ca²⁺ signaling pathways (Iriti and Faoro 2008; Iriti and Varoni 2015; Iriti et al. 2006; Lu et al. 2010; Zhao et al. 2007).

Polysaccharide peptide (PSP) is a macromolecule glycopeptide polymer in the mycelium of the Cov-1 strain of *Trametes versicolor*, a fungus in the polyporaceae family. PSP consists of 10% polypeptide and 90% polysaccharide, and its approximate molecular size is 100 kDa (Yeung and Or 2012). PSP has a stable chemical structure and is soluble in water. Thus, it can be easily extracted and efficiently purified by liquid fermentation at low cost. The exogenous application of PSP induces the production of superoxide dismutase that results in improved resistance of the human body to breast cancer, lung cancer, and some digestive cancers (Cui and Chisti 2003; Yeung and Or 2012).

We previously reported that the exogenous application of PSP (500 µg ml⁻¹), which was extracted from *T. versicolor*, exerts significant inhibitive effects on TMV infection in *N. tabacum* (Zhao et al. 2015). However, the mechanism underlying the enhanced virus resistance of PSP remained unclear. Our goal in this study was to reveal the mechanism involved in this antiviral action by the exogenous application of PSP to *Arabidopsis thaliana*.

RESULTS

Cucumber mosaic virus (CMV) activity in PSP-treated *A. thaliana*.

A. thaliana plants were inoculated with CMV after 72 h of PSP treatment. At 3 days after inoculation, the expression levels of the coat protein (CP) gene and protein of CMV were measured using real-time fluorescence-based quantitative polymerase chain reaction (qRT-PCR) and dot enzyme-linked immunosorbent assay (dot-ELISA) methods. The qRT-PCR test found that the relative mRNA levels of CMV decreased to 0.26 in the CMV-infected seedlings treated with a 500 µg ml⁻¹ PSP solution. This value was significantly lower than the levels of 0.46 and 0.68 in those treated with PSP concentrations of 100 and 300 µg ml⁻¹, respectively (Fig. 1A). Virus concentrations analyzed by dot-ELISA in the CMV-infected seedlings treated with different PSP concentrations showed similar patterns as those analyzed by qRT-PCR, with the lowest virus titer found in the seedlings treated with PSP at 500 µg ml⁻¹ (Fig. 1B). The treated and control *A. thaliana* plants challenged with CMV are shown in Supplementary Figure S1. These results indicate that PSP treatment can improve the antiviral ability of *A. thaliana* against the CMV virus.

PSP-induced Ca²⁺ influx.

To determine the role of Ca²⁺ signaling in the PSP induction of virus resistance, the Ca²⁺ influx was measured in *A. thaliana* plants after treatments with PSP, chitin, PSP + EGTA, and chitin + EGTA, using a Fluo 4-AM Ca²⁺ fluorescent probe. Significant green fluorescence was observed in *A. thaliana* cells after PSP and chitin treatments (Figs. 2A and B), while no fluorescence was observed in PSP + EGTA and chitin + EGTA groups (Figs. 2C and D) and the control group (water treatment) (Fig. 2E). These results demonstrated that EGTA inhibited PSP-induced Ca²⁺ influx.

PSP-induced H₂O₂ accumulation.

To investigate whether PSP induces H₂O₂ accumulation, the leaves of *A. thaliana* plants treated with PSP were stained with 3,3'-diaminobenzidine (DAB) and were observed under a microscope. The color yellow was easily observed in most of the PSP-treated plant cells (Fig. 3A). The control samples showed no such color reaction (Fig. 3B). This result indicated increased H₂O₂ accumulation in the leaves of *A. thaliana* following PSP treatment.

PSP-induced salicylic acid (SA) contents.

The SA content in the PSP treatment group was three times higher than that in the water treatment group in *A. thaliana* (Fig. 4). This result suggested that PSP-enhanced SA accumulation, thereby improving resistance to virus in *A. thaliana*.

PSP-induced transcriptome in *A. thaliana*.

Transcriptome analysis showed significant differences in the expression levels of 953 genes between the PSP-treated *A. thaliana* plants and the control plants. Among the 953 genes, 717 were up-regulated and 236 were down-regulated (Supplementary Fig. S2). Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) demonstrated that these genes were significantly up-regulated and enriched in pathways including their defense, immune system processes, and immune response (Supplementary Fig. S3). The enrichment analysis of these DEGs on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway revealed that numerous genes that were involved in Ca²⁺ signaling, reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK), and SA signaling exhibited significant upregulation (Supplementary Fig. S4). In addition, some genes with nucleotide binding site-leucine rich repeat (NBS-LRR) functional domains showed significant upregulation.

Analysis of the PSP-induced NBS-LRR functional domain.

After the T-DNA insertion mutation of the AT4G14370 gene (with a toll interleukin 1 receptor-NBS-LRR domain, referred

Fig. 1. Content of the *Cucumber mosaic virus* (CMV) virus in *Arabidopsis thaliana* measured using **A**, real-time fluorescence-based quantitative polymerase chain reaction and **B**, dot enzyme-linked immunosorbent assay after polysaccharide peptide (PSP) treatment. Data are presented as the means $\pm$ standard error. Different letters indicate significant differences at $P \leq 0.05$ by Student's t test. The shading represents the content of the virus, with dark indicating high virus content. P = positive control and N = negative control.

to as *LRR*), the expression level of the *LRR* gene was significantly down-regulated. In addition, the CMV virus content stopped decreasing when treated with PSP compared with that of PSP-treated wild-type *A. thaliana*. Concurrently, the expression level

Fig. 2. Polysaccharide peptide (PSP)-induced Ca^{2+} influx of *Arabidopsis* cells. **A**, PSP treatment, **B**, chitin treatment as positive control, **C**, PSP + EGTA treatment, **D**, chitin + EGTA treatment, and **E**, water treatment as negative control. **A**, **B**, **C**, **D**, and **E** show fluorescence images; **A1**, **B1**, **C1**, **D1**, and **E1** show bright images. Bars = 500 μm .

of the *PR1* gene was no longer significantly up-regulated when treated with PSP compared with that of PSP-treated wild-type *A. thaliana* (Fig. 5).

The transcriptome of the PSP-treated *LRR* gene-deletion mutants was compared between PSP-treated wild-type *A. thaliana* plants and the wild-type *A. thaliana* control plants. The results indicated that, after the deletion of the *LRR* gene in PSP-treated *A. thaliana*, 639 genes were down-regulated and 372 genes were up-regulated, compared with those of PSP-treated wild-type *A. thaliana* (Supplementary Fig. S5). A total of 574 genes were down-regulated and 522 genes were up-regulated in PSP-treated *A. thaliana*, compared with those of the wild-type *A. thaliana* after water treatment (Supplementary Fig. S6). A cluster analysis and a Venn diagram of the DEGs are shown in Supplementary Figures S7 and S8, respectively. The GO enrichment analysis of the DEGs in the *LRR* gene-deletion mutants and the wild-type *A. thaliana* after PSP treatment demonstrated that these genes exhibited significant down-regulation and enrichment in these pathways, including defense and immune responses (Supplementary Fig. S9). The enrichment analysis of the DEGs of the *LRR* gene-deletion mutants and the wild-type *A. thaliana* after PSP treatment on the KEGG pathway revealed that many genes involved in Ca^{2+} signaling, ROS, and MAPK exhibited significant upregulation (Supplementary Fig. S10). A total of 12 genes were selected. These genes were expressed significantly and differentially in wild-type *A. thaliana* and *LRR* gene-deletion mutants after PSP treatment. These genes were also involved in different pathways, including Ca^{2+} signaling, ROS, and MAPK. The expression levels of these genes in comparison with those in the water-treated wild-type *A. thaliana* were verified using qRT-PCR. The results were similar to those obtained from the transcriptome (Supplementary Fig. S11). These results indicate that the *LRR* gene is an important disease-resistant gene regulated by PSP to enhance the resistance of *A. thaliana*.

Characteristics of the *LRR* gene including phylogenetic analysis, gene subcellular localization, and tissue-specific expression are shown in Supplementary Figures S12, S13, and S14, respectively.

PSP-induced antiviral ability in *A. thaliana* depends on the Ca^{2+} influx by upregulating the *LRR* gene and increasing SA content.

The qRT-PCR method was used to measure the expression level of the *LRR* gene in wild-type *A. thaliana* after the respective PSP, PSP + EGTA (ethyleneglycol-bis (beta-aminoethylether)-*N,N'*-tetraacetic acid), and EGTA treatments and in the *sid2* mutants after PSP treatment. The results showed that PSP can significantly induce the upregulation of the *LRR* gene, while the PSP + EGTA and EGTA treatments alone cannot induce a significant upregulation of the *LRR* gene (Fig. 6). This phenomenon indicated that the presence of EGTA can block Ca^{2+} influx, thereby preventing PSP-induced upregulation of the *LRR* gene.

Wild-type *A. thaliana* was treated with PSP, SA, and PSP + EGTA. The *LRR* deletion mutants and the *sid2* mutants were treated with PSP. After these treatments, the *A. thaliana* seedlings were inoculated with the CMV virus. The relative levels of the CMV virus in *A. thaliana* after each treatment were measured using qRT-PCR and dot-ELISA. The water treatment group was used as a control group. The qRT-PCR results showed that PSP and SA can significantly reduce the content of the CMV virus in wild-type *A. thaliana* leaves, while PSP + EGTA cannot significantly influence the content of CMV virus in wild-type *A. thaliana*. In addition, PSP treatment of *LRR* deletion mutants and *sid2* mutants cannot significantly reduce the content of the CMV virus in *A. thaliana* mutants (Fig. 7).

The dot-ELISA test results were similar to those obtained using the qRT-PCR test (Supplementary Fig. S15).

The SA contents in the PSP-treated wild-type *A. thaliana*, PSP + EGTA-treated wild-type *A. thaliana*, and PSP-treated *LRR* deletion mutants and the overexpression of the *LRR* gene in *N. benthamiana* were measured, using high-performance liquid chromatography-mass spectrometry (HPLC-MS). Figure 8 shows that PSP treatment can significantly increase the SA content in wild-type *A. thaliana*. The SA content in wild-type *A. thaliana* treated with PSP + EGTA was significantly lower than that treated solely with PSP. The SA content in the *LRR* gene-deletion mutants after the PSP treatment was also significantly lower than that in wild-type *A. thaliana* after the PSP treatment. After heterologous overexpression of the *LRR* gene in *N. benthamiana*, the SA content in *N. benthamiana* was significantly higher than that in the control group.

When the heterologous overexpression of the *LRR* gene occurred in *N. benthamiana*, the relative expression level of the *PR1* gene and the relative content of CMV virus in *N. benthamiana* were determined using qRT-PCR. The results demonstrated that, when the *LRR* gene was overexpressed in *N. benthamiana*, relative expression of the *PR1* gene in *N. benthamiana* was up-regulated approximately eightfold (Supplementary Fig. S16), while the content of the CMV virus decreased by approximately 0.5-fold.

DISCUSSION

Little is known about the molecular mechanism of the anti-plant virus action of PSP. In this study, the model plant *A. thaliana* was used to investigate the antiviral mechanism involved in the induction of virus resistance by exogenous application to *A. thaliana*. The results showed that PSP-induced antiviral effects depend on the Ca^{2+} influx by increasing the SA content and upregulating the *LRR* gene in *A. thaliana*.

Ca^{2+} signaling is a second important messenger that mediates the signaling of various abiotic and biotic stresses, including those of viruses (Garcia-Brugger et al. 2006; Yuan et al. 2017). Ca^{2+} signaling plays an important role in plant immune responses (Choi et al. 2017; Gilroy et al. 2016). Many proteins may relay and disturb Ca^{2+} signaling by producing activated

phosphorylation events and rapid bursts of ROS and these proteins may also induce massive transcriptional reprogramming and synthesize defense-related phytohormones, such as SA, jasmonic acid, and ethylene (Cui et al. 2015; Du et al. 2009). Many other studies have reported the effect of Ca^{2+} signaling on plant immune responses. For example, Lecourieux et al. (2002) found that pathogen elicitors can stimulate the influx of Ca^{2+} and H_2O_2 accumulation in *N. tabacum* cells, which play vital roles in improving the resistance of *N. tabacum* to pathogens. Grant et al. (2000) revealed that the *RPM1* gene of *A. thaliana* can promote Ca^{2+} influx in *A. thaliana* cells and further induce oxidative bursting and allergic necrosis of host cells, thereby enhancing plant resistance to pathogens. Gerber et al. (2004) indicated that lipopolysaccharides can enhance the

Fig. 4. Increased salicylic acid (SA) content in *A. thaliana* induced by polysaccharide peptide (PSP) treatment. CK = water treatment. Data are presented as the means $\pm$ standard error.

Fig. 3. Polysaccharide peptide (PSP)-induced H_2O_2 accumulation in *Arabidopsis thaliana* leaves. **A**, PSP treatment; **B**, Water treatment. Bars = 50 μm .

resistance of plants to pathogenicity by activating plant Ca^{2+} signaling. In plant antiviral reactions, Ca^{2+} influx plays important roles in improving the resistance of plants to viruses. For example, Lu et al. (2010) demonstrated that the *N. tabacum* Ca^{2+} influx induced by oligochitosan is critical for the resistance of *N. tabacum* to TMV virus. Laminarin sulfate can also induce Ca^{2+} influx in *N. tabacum* cells, which enhances the resistance of *N. tabacum* to the TMV virus (Ménard et al. 2004). Our study found that PSP treatment can significantly increase the Ca^{2+} influx in *A. thaliana* cells, which is vital for the virus resistance of *A. thaliana*.

H_2O_2 accumulation in plant cells also plays an important role in improving the resistance of host plants to pathogens (Baxter et al. 2014). *RBOH* genes are a critical source of H_2O_2 , which is the most stable type of ROS (Gechev and Hille 2005). In *Arabidopsis*, *rbohD* and *rbohF* mutants reduce ROS generation when they are infected with *Pseudomonas syringae* DC3000 (Kwak et al. 2003). In *N. benthamiana*, ROS production is impaired when *RBOHA* and *RBOHB* are silenced. Thus, the resistance to infection with *Phytophthora infestans* is compromised (Yoshioka et al. 2003). In plant antiviral reactions, H_2O_2 accumulation in host plant cells is also crucial in improving the resistance of plants to viruses. The brassinosteroid-induced H_2O_2 production is associated with systemic virus resistance (Deng et al. 2016). Laminarin sulfate induces oxidative bursts in *N. tabacum* cells, which enhances the resistance of *N. tabacum* to the TMV virus (Ménard et al. 2004). Our study found that PSP treatment can significantly increase H_2O_2 accumulation in *A. thaliana* cells.

The SA signaling pathway is important for plants to resist various biotic and abiotic stresses (Delaney et al. 1994; Lewsey and Carr 2009; White 1979). White (1979) found that SA can improve the resistance of *N. tabacum* to the TMV virus. Delaney et al. (1994) revealed that SA plays an important role in the disease resistance of plants. Chivasa et al. (1997) also reported that SA induces the inhibition of TMV replication by inhibiting the accumulation of TMV RNA, CP, and viral RNA-dependent RNA polymerase activity in virus-inoculated leaves of a nn genotype tobacco. In this study, we found that PSP can

significantly increase the SA content in *A. thaliana*. This result indicated that increasing SA levels can be considered a defense pathway involved in PSP-mediated resistance to the virus.

During the long-term synergistic evolution of plants and pathogens, plants have evolved complex mechanisms to resist pathogens (Jones and Dangl 2006). When plants are attacked by pathogens, the conserved pathogen-associated molecular pattern molecules on the surface of pathogens are identified by transmembrane receptors on the cell surface to stimulate the basal defense response of plants and resist the invasion of pathogens. To infect plants continuously, pathogens will secrete an effector protein into the plant cells to interfere with or inhibit the host PTI reaction. Furthermore, the plants will possess their own disease-resistant proteins to identify these effector proteins of the pathogens specifically and resist the infection. Thus, the downstream disease resistance of plants is activated, and the protein-induced defense response is triggered (Jones and Dangl 2006; Liu et al. 2013). To date, scientists have found many resistance (R) genes in *A. thaliana*, *Nicotiana glutinosa*, *N. benthamiana*, *Hordeum vulgare*, *Solanum lycopersicum*, *Capisicum annuum*, *Oryza sativa*, *Linum usitatissimum*, and *Solanum tuberosum* (Caplan et al. 2008). Most of these R genes possess conserved NBS-LRR functional domains that play vital roles in the disease-resistant response of the host (Caplan et al. 2008). In this study, we selected the genes with an NBS-LRR functional domain that exhibited significant PSP-induced upregulation through transcriptome sequencing. An *LRR* gene was obtained by additional screening of the *A. thaliana* deletion mutants of these genes. After the deletion mutation of this gene, the PSP-induced response of *A. thaliana* was significantly attenuated. The SA signaling pathway of *A. thaliana* regulated by the *LRR* gene was further confirmed to affect the resistance of *A. thaliana* by measuring the SA content and the expression level of the *PR1* gene in the *LRR* gene-deletion mutants in *A. thaliana* after PSP treatment.

In conclusion, this study explained the mechanism of the PSP-induced resistance of *A. thaliana* to the CMV virus. PSP enhanced the antiviral resistance of *A. thaliana* by inducing Ca^{2+} influx and accumulating H_2O_2 . Subsequently, PSP upregulated the *LRR* gene, which further increased the SA content. H_2O_2 synthesis may also influence the upstream regulation to

Fig. 5. Influence of polysaccharide peptide (PSP), water-treated wild-type *Arabidopsis thaliana*, and two lines of toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat domain (*LRR*) deletion mutants of *A. thaliana* on the expression levels of *Cucumber mosaic virus* and *PR1* gene measured using real-time fluorescence-based quantitative polymerase chain reaction. Col_W = wild-type *Arabidopsis* treated with water; Col_P: wild-type *Arabidopsis* was treated with 500 $\mu\text{g ml}^{-1}$ PSP; LRR_W_L1 = *LRR* line 1 mutant *Arabidopsis* treated with water; LRR_P_L1 = *LRR* line 1 mutant *Arabidopsis* treated with 500 $\mu\text{g ml}^{-1}$ PSP; LRR_W_L2 = *LRR* line 2 mutant *Arabidopsis* treated with water; LRR_P_L2 = *LRR* line 2 mutant *Arabidopsis* treated with 500 $\mu\text{g ml}^{-1}$ PSP. Data are presented as the means $\pm$ standard error. Different letters indicate significant differences at $P \leq 0.05$ by the Student's *t* test.

Fig. 6. Relative expression level of the leucine-rich repeat (*LRR*) gene after polysaccharide peptide (PSP), PSP + EGTA, and EGTA treatments. The wild-type *Arabidopsis thaliana* was treated with 500 $\mu\text{g ml}^{-1}$ PSP (Col_PSP), 500 $\mu\text{g ml}^{-1}$ PSP + 2 mM EGTA (Col_PSP+EGTA), and 2 mM EGTA (Col_EGTA). The *sid2* mutants were treated with 500 $\mu\text{g ml}^{-1}$ PSP (Sid2_PSP), 500 $\mu\text{g ml}^{-1}$ PSP + 2 mM EGTA (Sid2_PSP+EGTA), and 2 mM EGTA (Sid2_EGTA). Data are presented as means $\pm$ standard error. Different letters indicate significant differences at $P \leq 0.05$ by the Student's *t* test.

increase cytosolic Ca^{2+} concentrations (Lecourieux et al. 2006; Vandelle et al. 2006). Upregulation of the *LRR* gene depends on Ca^{2+} influx. Additionally, Ca^{2+} influx has been shown to induce SA accumulation (Wang et al. 2009b). Similarly, the *LRR* gene upregulation may also further increase the SA content. Thus, the antiviral resistance of plants was improved. Substantial PSP can be obtained by fermentation of the *T. versicolor* spores. This method is cost-effective, with simple extraction techniques and without subjection to external environmental conditions. In addition, PSP is highly active, safe for humans and animals, and is inexpensive to prepare. Therefore, this polymer possesses significant potential for commercial agricultural applications.

MATERIALS AND METHODS

Plant material.

A. thaliana mutants AT4G14370 (SALK_149141c, line 1; SALK_047576.56, line 2), AT5G22690 (SALK_061751c), AT4G19520 (SALK_118667c), AT5G41750 (SALK_133292), AT1G74360 (SAIL_859_H01), and AT1G15890 (SALK_055219) with an NBS-LRR functional domain, the SA-deficient mutant *sid2*, and the wild-type Col-0 *A. thaliana* were obtained from the Arabidopsis Biological Resource Center (ABRC) (The Ohio State University, Columbus, OH, U.S.A.). *A. thaliana* seeds were surface-disinfected as described by Faigón-Soverna et al. (2006). Subsequently, the seeds were induced by maintenance at 4°C in the dark for 3 days. The seeds were sown in pots (6 cm) that contained peat (Pindstrup Substrate). They were grown for seedling production in a net greenhouse at a light intensity of $225 \mu\text{mol s}^{-1}\text{m}^{-2}$ provided with Philips LED lamps under a 16-h photoperiod at 24°C day and 20°C of night temperatures. *A. thaliana* mutants were tested using primers provided on the ABRC website. When homozygotes were detected, their seeds were resown and were subjected to different treatments at week 4 of the experimental study.

CMV inoculation method.

CMV strain Fny was provided by the State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University (Yangling, China) in the form of virus-infected plants of *N. tabacum* L. cv. K326. The CMV inoculation method was based on the inoculation method described by Ryu et al. (2004). Briefly,

Fig. 7. Relative contents of the *Cucumber mosaic virus* in *A. thaliana* after different treatments measured by real-time fluorescence-based polymerase chain reaction quantitative determination. The wild-type *A. thaliana* was treated with concentrations of $500 \mu\text{g ml}^{-1}$ polysaccharide peptide (PSP) (Col_P), 1 mM salicylic acid (SA) (Col_SA), and $500 \mu\text{g ml}^{-1}$ PSP + 2 mM EGTA (Col_P+EGTA). Two lines of *LRR* deletion mutants were treated with $500 \mu\text{g ml}^{-1}$ PSP (LRR_P_L1 and LRR_P_L2), and the *sid2* mutants were treated with $500 \mu\text{g ml}^{-1}$ PSP (sid2_P). Data are presented as means $\pm$ standard error. Different letters indicate significant differences at $P \leq 0.05$ by the Student's *t* test.

1 g of CMV-infected *N. tabacum* L. cv. K326 leaves was mixed with 10 ml of potassium phosphate buffer (50 mM, pH 7.0) and sodium sulfite (10 mM). The mixture was completely ground, was filtered through gauze, and was stored at 4°C, for future use. The *A. thaliana* plants were left to grow for approximately 4 weeks. Afterward, three old leaves on the lower part of the plant were inoculated with freshly prepared CMV virus inoculum.

Observation of $[\text{Ca}^{2+}]_{\text{cyt}}$ by fluorescence microscope.

The main veins were removed from *A. thaliana* leaves. The leaves were added to MES-Tris buffer (Wu and Wu 2008) (0.2 mM CaCl_2 , 50 mM sorbitol, and $1 \mu\text{M}$ fluo 4-AM, pH, 6.1) and were placed in darkness for 45 min. The leaves were washed three times with Tris-KCl buffer (Tris 10 mM, KCl 50 mM, pH 7.2) and were placed in Tris-KCl buffer that contained concentrations of $500 \mu\text{g ml}^{-1}$ PSP and $500 \mu\text{g ml}^{-1}$ PSP with the calcium chelator EGTA (2 mM). A separate Tris-KCl buffer treatment was used as the negative control group. Leaves were observed using confocal laser scanning microscopy (LSM 510 META; Zeiss) with excitation light at 495 nm and emitted light at 515 nm.

Observation of H_2O_2 staining.

The H_2O_2 content in *A. thaliana* leaves was detected by DAB chemical staining (Herzog and Fahimi 1973). After the leaves were treated with a concentration of $500 \mu\text{g ml}^{-1}$ PSP for 12 h, they were immersed in a 1-mg ml^{-1} solution of DAB in Tris-acetate buffer (50 mM, pH 6.5) for 8 h at room temperature. Chlorophyll in the leaves was removed using 95% ethanol and were observed using a microscope (BX51, Olympus). Another group was treated with water as a control group.

Determination of the SA content by HPLC-MS.

After *A. thaliana* plants were treated with $500 \mu\text{g ml}^{-1}$ PSP spray for 3 days, SA was extracted, as described by Verberne et al. (2002), and was analyzed by HPLC-MS. All the solutions were filtered through $0.22 \mu\text{m}$ membranes prior to analysis, as

Fig. 8. Salicylic acid (SA) content in *Arabidopsis thaliana* after different treatments, according to high-performance liquid chromatography-mass spectrometry results. Col_W = wild-type *A. thaliana* with water treatment; Col_P = wild-type *A. thaliana* with $500 \mu\text{g ml}^{-1}$ polysaccharide peptide (PSP) treatment; Col_P + EGTA = wild-type *A. thaliana* with $500 \mu\text{g ml}^{-1}$ PSP + 2 mM EGTA treatment; LRR_P_L1 = leucine-rich repeat (*LRR*) line 1 deletion mutants with $500 \mu\text{g ml}^{-1}$ PSP treatment; LRR_P_L2 = *LRR* line 2 deletion mutants with $500 \mu\text{g ml}^{-1}$ PSP treatment; NB_LRR = tobacco leaves when the *LRR* gene was overexpressed for 3 days; and NB_GFP = tobacco leaves when the green fluorescent protein gene was overexpressed for 3 days. Data are presented as the means $\pm$ standard error. Different letters indicate significant differences at $P \leq 0.05$ by the Student's *t* test.

described by Jia et al. (2016). A total of 10 µl of extract was injected into the analytical column.

Chromatographic analysis was carried out using an ACQUITY UPLC system (Waters) equipped with a cooling autosampler and column oven, as described by Jia et al. (2016). A C18 column (Termo, 250 × 4.6 mm) with a security guard column was used for the HPLC separations. The column temperature was maintained at 30°C, and the autosampler was kept at 4°C. The mobile phase consisted of solvents A (5 ml of formic acid per liter) and B (methyl alcohol) at a flow rate of 1 ml min⁻¹. Mass spectrometric detection was performed using a Micromass API 2000 LC/MS/MS (AB Sciex) with an electrospray ionization source, as described by Jia et al. (2016). The calibration data for each component were obtained with linear regression by a graph representing the area ratio of an external standard against the known concentration of the external standard (Wang et al. 2014).

Expression analysis.

qRT-PCR and dot-ELISA were used to analyze the gene and viral protein expression. Dot-ELISA was conducted as described by Yu et al. (2002), using a commercial kit (Neogen) according to the manufacturer instructions. qRT-PCR was performed as described by Chen et al. (2008). In brief, total RNA was extracted from the leaves of the inoculated seedlings as described by Zhao et al. (2015). After purification, the RNA was diluted to 200 ng/µl using double-distilled water. cDNA reverse transcription was performed using a PrimeScript RT reagent kit (Takara) according to the manufacturer instructions. The qRT-PCR test was conducted using a CFX1000 (Bio-Rad) instrument and a SYBR Premix ExTaq II kit (Takara) reagent. All primers used for the qRT-PCR are shown in Supplementary Table S1. The relative expression levels of each gene were normalized to the expression of the actin gene. Values are the mean ± standard error of three biological repeats.

RNA-seq analysis.

Our preliminary study found that the expression level of the *PR1* gene reached the highest value at 3 days of PSP treatment. Therefore, in this study, the transcriptome was measured in *A. thaliana* whole plants that were treated with PSP once for 3 days. Afterward, the wild-type and the *LRR* gene-deletion mutant line 1 *A. thaliana* plants were treated with 500-µg ml⁻¹ PSP and water spray, respectively. Five *A. thaliana* plants with identical treatments were mixed as a biological repeat, with three repeats in each group. Total RNA was extracted using the TRIzol reagent (Invitrogen). Library construction was performed according to Illumina instructions and was sequenced on a HiSeq 2000 sequencer. Raw data (raw reads) in FASTQ format were first processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low-quality reads from raw data. Additionally, the Q20, Q30, and GC contents of the clean data were calculated. All the downstream analyses were based on clean data of high quality. All paired-end reads were mapped to the *Arabidopsis* genome using TopHat2 (Kim and Salzberg 2011). The expression level of the gene was calculated using the reads per kilobase per million reads method (Mortazavi et al. 2008). The DESeq R package (1.10.1) was used to analyze the DEGs between differently treated samples. The DEGs were filtered for a corrected *P* value threshold of 0.005 (*P* values were adjusted using the Benjamini and Hochberg method). A corrected *P* value of 0.005 and log₂ (fold change) of 1 were set as the threshold for significantly differential expression. We subsequently used the DAVID Bioinformatics Resources 6.7 web tool (Huang et al. 2008, 2009) to assign the clustered genes to biological process categories based on the GO analysis. The significantly enriched GO terms for the DEGs were identified using a hypergeometric test by

comparison with the genomic background (corrected *P* = 0.05). GO terms with a corrected *P* value of less than 0.05 were considered significantly enriched by DEGs. KEGG pathway-based analysis was carried out using the blastall program against the KEGG database. Significantly enriched metabolic pathways or signal transduction pathways for the DEGs were identified using pathway enrichment analysis (corrected *P*=0.05; Kanehisa et al. 2008). Sequence data from this article can be found in the National Center for Biotechnology Information Short Read Archive sequence database under accession number SRS2319784.

Analysis of the PSP-induced NBS-LRR functional domain.

The NBS-LRR functional domain is a common functional domain of virus-resistant genes (Caplan et al. 2008). Therefore, six genes with an NBS-LRR functional domain that were the most significantly up-regulated after induction with PSP were selected and analyzed. The deletion mutants of these six genes were purchased. The deletion mutants were treated with PSP and were inoculated with CMV virus after 3 days. The expression levels of the CMV virus *CP* gene and the *PR1* gene in *A. thaliana* were determined by qRT-PCR after inoculation.

Heterologous expression of the *LRR* gene in *Nicotiana benthamiana*.

The *LRR* gene was constructed in pCambia1302 plasmid-transformed *Agrobacterium tumefaciens* and injected into *N. benthamiana*. *Agrobacterium tumefaciens* transformed by pCambia1302 plasmid without the *LRR* gene was injected as the negative control group. After 48 h, the CMV virus was inoculated on the leaves of injected *N. benthamiana*. After 3 days, the *PR1* gene expression levels in the CMV-infected *N. benthamiana* were determined by qRT-PCR as described above.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- DAVID Bioinformatics Resources 6.7 web tool:
<http://david.abcc.ncifcrf.gov/home.jsp>
- Kyoto Encyclopedia of Genes and Genomes (KEGG) database:
<http://www.genome.jp/kegg>