Virology 527 (2019) 122–131

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Immune responses induced by different genotypes of the disease-specific protein of *Rice stripe virus* in the vector insect

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ARTICLE INFO

Keywords:
Rice stripe virus
Single nucleotide polymorphism
Small brown planthopper
Rice
Disease-specific protein
Immune reaction

ABSTRACT

Persistent plant viruses circulate between host plants and vector insects, possibly leading to the genetic divergence in viral populations. We analyzed the single nucleotide polymorphisms (SNPs) of *Rice stripe virus* (RSV) when it incubated in the small brown planthopper and rice. Two SNPs, which lead to nonsynonymous substitutions in the disease-specific protein (SP) of RSV, produced three genotypes, i.e., GG, AA and GA. The GG type mainly existed in the early infection period of RSV in the planthoppers and was gradually substituted by the other two genotypes during viral transmission. The two SNPs did not affect the interactions of SP with rice PsbP or with RSV coat protein. The GG genotype of SP induced stronger immune responses than those of the other two genotypes in the pattern recognition molecule and immune-responsive effector pathways. These findings demonstrated the population variations of RSV during the circulation between the vector insect and host plant.

1. Introduction

Because of the error-prone replication characteristics of the RNA-dependent RNA polymerase (RdRp), RNA viruses are usually comprised of a diverse mixture of virus mutants called as quasispecies or viral swarms (Elena and Sanjuan, 2005; Rozen-Gagnon et al., 2014). The high mutation rate and the large viral population improve the virus fitness for adapting to new environmental conditions. Most persistent plant viruses and arboviruses circulate between hosts (plants and humans) and vector insects. The large contrast in living conditions imposes differential selective pressures on viral populations, leading to the genetic divergence in viral populations during the transmission between hosts and vector insects.

Plant viruses often induce serious or fatal diseases in hosts, while they usually cause asymptomatic and harmless infections in vectors (Ramirez and Haenni, 1994). Persistent-propagative plant viruses have a limited replication level in the vector insect and attain a balance between viral replication and surveillance of the insect immune system. Persistent-propagative plant viruses activate the immune response through the Toll pathways, pathogen recognition molecules, reactive oxygen, immune-responsive effectors, and JNK signaling pathway in the vector insects (Medeiros et al., 2004; Wang et al., 2017; Zhao et al., 2016a). The genetic variation, or selection of different viral quasispecies, during viral incubation in vector insects helps the virus adapt to the insect immune system.

Rice stripe virus (RSV), a single-stranded RNA virus of the genus *Tenuivirus*, is a persistent-propagative plant virus that is efficiently transmitted by the small brown planthopper, *Laodelphax striatellus*, in both horizontal and vertical manners (Falk and Tsai, 1998). The genome of RSV contains four RNA segments (Mami Takahashi et al., 1993; Shigemitsu Toriyama et al., 1994). RNA1 is negative-sense and encodes the RNA-dependent RNA polymerase (RdRp). The other three segments are ambisense and encode NS2 (nonstructural proteion with unknown function), NSvc2 (putative membrane glycoprotein), NS3 (gene silencing suppressor), CP (nucleocapsid protein), SP (disease-specific protein), and NSvc4 (movement protein) (Cho et al., 2013). RSV causes serious stripe disease in rice, while it is almost harmless to the vector insect (Toriyama, 1986; Wang et al., 2005). Different selective pressure may result in the diversity of viral quasispecies in RSV populations during the alternation between vector insects and host

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https://doi.org/10.1016/j.virol.2018.11.011
Received 3 October 2018; Received in revised form 15 November 2018; Accepted 17 November 2018
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plants. To explore this hypothesis, we sequenced the RSV genomes in viruliferous *L. striatellus* and RSV-infected rice and performed *de novo* genome assembly. Sixteen- and 15-nt extension sequence variations at the 3′-termini of the RNA1 and RNA2 segments have been observed (Zhao et al., 2018). Here, we continued to analyze the *de novo* assembled RSV genomes and reported single nucleotide polymorphisms (SNPs) of the RSV populations in vector insects and host plants. Especially three genotypes of viral SP from two SNP sites were further explored for the adaptation of RSV to the insect immune system.

2. Materials and methods

2.1. Virus and culturing of insects and plants

The viruliferous and nonviruliferous small brown planthopper populations used in this study were established from a field population collected in Hai'an, Jiangsu Province, China, in 2009. This viruliferous strain contained the Jiangsu isolate of RSV. The planthoppers were reared separately on 2–3 cm seedlings of rice, *Oryza sativa* Huangjinqing, in glass incubators at 25 °C with 16 h of light daily.

2.2. Sample preparation for transcriptome sequencing

Non-viruliferous fourth-instar planthoppers were fed on an artificial diet containing the RSV crude preparations from the infected rice leaves as previously described (Zhao et al., 2016b). After feeding on RSV for 4 h, the nymphs were transferred to healthy rice seedlings and then collected after 5 d when the planthoppers grew to the fifth-instar nymphs. The corresponding fifth-instar nymphs from the viruliferous population were also collected. Both of the two group samples and 20 planthoppers per group were applied for transcriptome sequencing.

Viruliferous fifth-instar planthoppers were transferred to fresh rice seedlings for RSV inoculation. After 24 h feeding, the planthoppers were removed and the inoculated seedlings were cultured for 15 d until disease symptom appeared. Each rice seedling was fed by five viruliferous planthoppers, and three seedlings were used in a replicate for transcriptome sequencing.

2.3. SNP analysis of RSV populations in small brown planthoppers and rice plants

The transcriptomes of the small brown planthoppers that had acquired the RSV 5 d prior, the viruliferous planthoppers, and the RSV-infected rice were deep sequenced in our previous work (Zhao et al., 2018). Two key steps of the transcriptome sequencing were depletion of rRNA and usage of random hexamers for first-strand cDNA generation (Zhao et al., 2018). Three replicates each were prepared for the viruliferous planthoppers and RSV-infected rice samples. One replicate was prepared for the planthoppers that had acquired RSV 5 d prior to sampling. Approximately 12 GB of 101-bp, paired-end raw data were generated for each library. The sequencing data were deposited on the Short Read Archive of the National Center for Biotechnology Information (NCBI) with the accession number SRP108307 for further SNP analysis.

Clean paired-end reads were mapped to the newly assembled RSV genome (MF287955.1 for RNA 1, MF287954.1 for RNA 2, MF287953.1 for RNA 3, MF287956.1 for RNA 4) (Zhao et al., 2018) by BWA software. Only those paired-end reads properly aligned to RSV genome were kept (samtools view-f 0 × 2). Two methods were utilized in the SNP calling process. The first one was using SAMtools and BCFtools (Li and Durbin, 2009). The reads properly aligned to RSV genome were sorted with SAMtools and SNPs were identified using mpileup function. Allele frequency at each genomic position was calculated according to the mpileup output by a custom python script. Mpileup output was then subjected to the call function of BCFtools to get the SNP information. VCF format files were transferred to the filter function of BCFtools to filter those low quality SNPs (a QUAL threshold of 30). The second method for SNP calling was using Genome Analysis Toolkit (GATK) (McKenna et al., 2010). The raw alignments were preprocessed by base quality score recalibration, removal of PCR duplicates, and indel realignment and then were used as the input for GATK. Only those SNPs called by both SAMtools and GATK were kept for downstream analysis. The functional effects of SNPs were evaluated with SnpEff (Cingolani et al., 2012).

2.4. RNA isolation and cDNA synthesis

Total RNAs were isolated from 5 fifth-instar viruliferous planthopper nymphs, 5 fourth-instar planthoppers nymphs after acquiring RSV for 5 d or from 100 mg of RSV-infected rice leaves (with the typical stripe symptom) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The concentration and quality of RNA was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and gel electrophoresis. RNA was treated using the TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove genomic DNA contamination before cDNA synthesis. One microgram of RNA was reverse transcribed to cDNA using the Superscript III First-Strand Synthesis System (Invitrogen) and random primers (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

2.5. SP gene cloning and sequencing

The full-length open reading frames (ORF) of the RSV *SP* gene were amplified with the primer pair *SP*-orf-*F*/*SP*-orf-*R* using cDNA samples obtained from viruliferous planthoppers, planthoppers after acquiring RSV for 5 d, and RSV-infected rice leaves (Supplemental Table 1). The PCR protocol was as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and finally 72 °C for 5 min. After purification with Wizard® SV Gel and PCR Cleanup Kit (Promega), the PCR products were subcloned into the pEASY-Blunt Zero vector (TransGen Biotech, Beijing, China). The ligation products were transformed into competent cells of *Escherichia coli* strain Trans T1 (TransGen Biotech). After PCR confirmation, the recombinant plasmids were sent to Sangon Biotech company (Beijing, China) for Sanger sequencing.

2.6. Protein expression and purification

Three genotypes of *SP* were subcloned into the pET28a vector between the NdeI-XhoI restriction sites with the primer pair *SP*-NdeI/*SP*-XhoI to generate the SP-His recombinant plasmids (Supplemental Table 1). The ORFs of RSV *CP* (DQ299151 in GenBank) and rice *PsbP* (KF460579 in GenBank) were amplified with the primer pairs *CP*-orf-*F*/*CP*-orf-*R* and *PsbP*-orf-F/*PsbP*-orf-R, respectively (Supplemental Table 1). After verification with Sanger sequencing, the ORF of *CP* or *PsbP* was subcloned into the pGEX-3X vector between the BamHI-SmaI restriction sites with the primer pair CP-BamHI-F/CP-SmaI-R or PsbP-BamHI-F/PsbP-SmaI-R to generate CP-GST or PsbP-GST recombinant plasmids, respectively (Supplemental Table 1). The recombinant plasmids were transformed into the *E. coli* strain BL21 (DE3) for protein expression. After 4 h induction with 0.4 mM isopropyl β-D-thiogalactoside at 37 °C, cells were pelleted by centrifugation and then were sonicated for 30 min at 4 °C. The expressed SP in the supernatant from the sonicated cells was purified using Ni Sepharose (GE Healthcare, Buckinghamshire, UK) following the manufacturer's instructions and tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentrations of purified SP proteins were determined with a BCA protein assay kit (Thermo Scientific). The expressed CP-GST and PsbP-GST in the supernatant were used for detection of interactions with SP proteins in pull-down experiments.

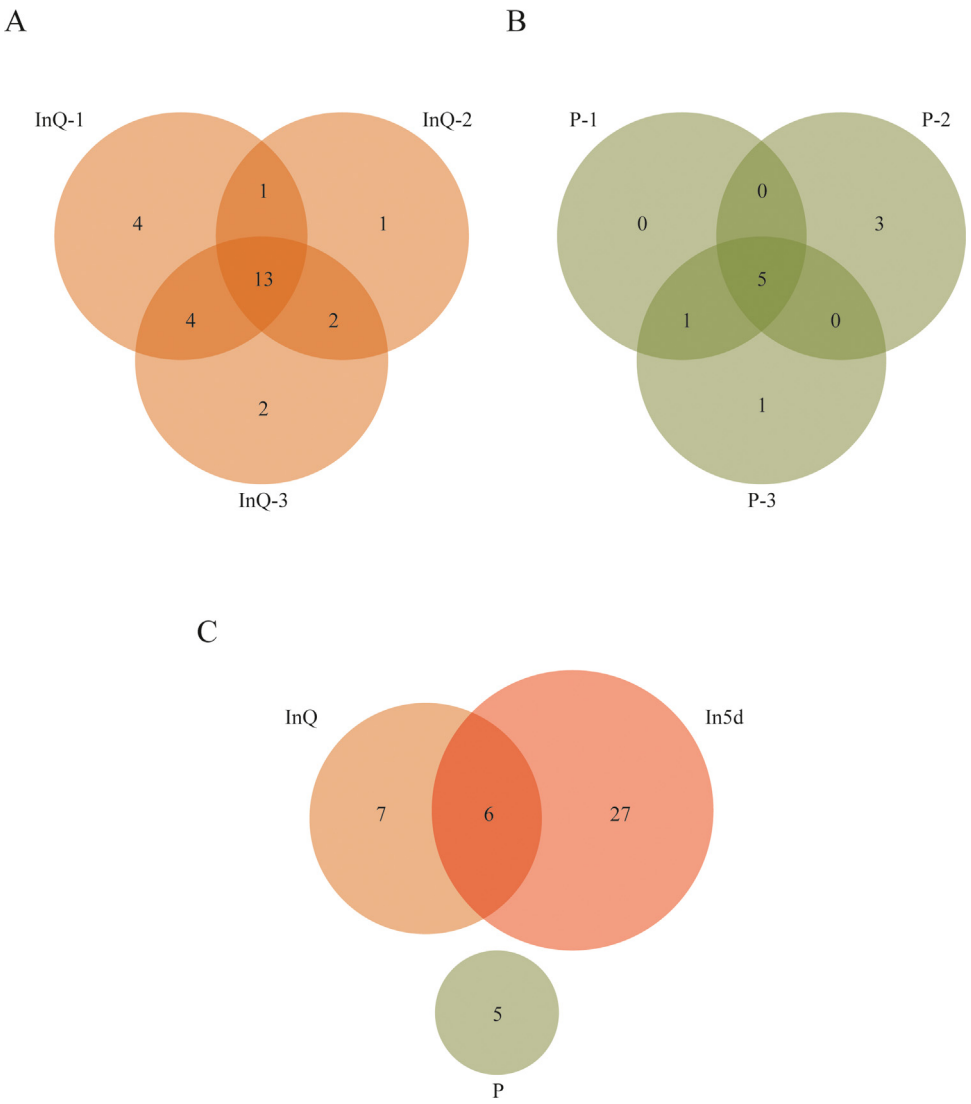

Fig. 1. Venn diagrams demonstrating the number of single nucleotide polymorphisms (SNPs) in the RSV genome in planthoppers and rice. (A) Overlap of SNPs identified in three replicates of viruliferous small brown planthoppers (InQ). (B) Overlap of SNPs identified in three replicates of RSV-infected rice leaves with obvious stripe symptom (P). (C) Overlap of SNPs identified in viruliferous small brown planthoppers, RSV-infected rice leaves, and the small brown planthoppers 5 d after acquiring RSV (In5d).

2.7. His-tag and GST-tag pull-down and western blot assay

SP-His recombinant proteins were bound to Ni Sepharose (GE Healthcare, Buckinghamshire, UK) and incubated for 2 h at 4 °C. The CP-GST recombinant proteins were added and incubated with SP-His recombinant protein for 2 h at 4 °C. After washed with lysis buffer (20 mM sodium phosphate containing 50 mM imidazole, pH 8.0), proteins were released using elution buffer (20 mM sodium phosphate containing 250 mM imidazole, pH 8.0), and separated by SDS-PAGE gel electrophoresis. The presence of target proteins was verified by western blot with anti-SP or anti-CP monoclonal antibody. Expression products from the pET28a vector and pGEX-3X vector were used as negative controls.

PsbP-GST recombinant proteins were bound to glutathione-Sepharose beads (GE Healthcare) for 2 h at 4 °C. SP-His recombinant proteins were added and incubated overnight at 4 °C. After centrifugation and five washes with wash buffer (140 mM NaCl, 10 mM Na_2HPO_3, 2.7 mM KCl, and 1.8 mM KH_2PO_4, pH 7.4), proteins were released using elution buffer (10 mM Glutathione in 50 mM Tris-HCl, pH 8.0), and separated by SDS-PAGE gel electrophoresis. The presence of target proteins was verified by western blot with an anti-SP monoclonal antibody or an anti-GST polyclonal antibody (CW Biotech, Beijing, China). Expression products from the pET28a vector and pGEX-3X vector were used as negative controls.

2.8. Injection of three genotypes of SP proteins in planthoppers

Purified SP recombinant proteins were microinjected into the hemolymph of nonviruliferous fourth instars in the ventral thorax using a Nanoliter 2000 instrument (World Precision Instruments, Sarasota, Florida, USA). A total of 23 nL of recombinant proteins of each genotype at 1.1 mg/mL were injected in individual insects. The insects injected with an equal volume of the purified products from the pET28a vector were used as mock group. After 24 h and 48 h, total RNA of each group was extracted and reverse transcribed to cDNA for further quantitative real-time PCR assay. Six to eight biological replicates with five insects per replicate were prepared.

2.9. Quantitative real-time PCR analysis of immune gene expression in planthoppers

The transcript levels of 19 immune genes were determined using

Table 1
Summary of single-nucleotide polymorphisms identified in the *Rice stripe virus* population in the small brown planthoppers and rice leaves.

Segment	Genome position	Gene ID	Nucleotide change	Amino acid change	Substitution Ratio						
					In5d	InQ-1	InQ-2	InQ-3	P-1	P-2	P-3
RNA1	219	RdRp	C– > T	Ser	0	0.64	0.63	0.62	1	1	1
RNA1	399	RdRp	T– > C	Ala	0	0	0	0	1	1	1
RNA1	440	RdRp	G– > A	Gly– > Asp	0.83	0	0	0	0	0	0
RNA1	1464	RdRp	G– > T	Glu– > Asp	0.65	0	0	0	0	0	0
RNA1	2274	RdRp	A– > T	Glu– > Asp	0.94	0	0	0	0	0	0
RNA1	3126	RdRp	A– > T	Val	0.66	0	0	0	0	0	0
RNA1	3649	RdRp	T– > G	Phe– > Val	0.24	0	0	0	0	0	0
RNA1	3963	RdRp	T– > C	Asp	0.69	0	0	0	0	0	0
RNA1	4942	RdRp	G– > A	Val– > Met	0.43	0	0	0	0	0	0
RNA1	5137	RdRp	G– > A	Val– > Met	0	0	0	0	1	1	0.97
RNA1	6646	RdRp	A– > G	Lys– > Glu	0	0.17	0.15	0.23	0	0	0
RNA1	6828	RdRp	T– > A	Thr	0.74	0	0	0	0	0	0
RNA1	7590	RdRp	A– > G	Lys	0.7	0	0	0	0	0	0
RNA1	7983	RdRp	C– > T	Tyr	0.68	0	0	0	0	0	0
RNA1	8425	RdRp	A– > G	Ser– > Gly	0.71	0	0	0	0	0	0
RNA2	25	5′-NTR	C– > T		0.21	0	0	0	0	0	0
RNA2	718	IR	C– > T		0.57	0	0	0	0	0	0
RNA2	901	NSvc2	A– > G	Leu	0.54	0	0	0	0	0	0
RNA2	951	NSvc2	C– > T	Cys– > Tyr	0.64	0	0	0	0	0	0
RNA2	1205	NSvc2	A– > G	Leu	0	0	0	0	0.96	0.96	0.96
RNA2	1723	NSvc2	C– > T	Asp– > Asn	0	0	0	0	0.21	0.36	0.19
RNA2	1796	NSvc2	G– > T	Ala	0.17	0	0	0	0	0	0
RNA2	2480	NSvc2	C– > T	Val– > Ile	0	0	0	0	0.56	0.55	0.64
RNA2	2728	NSvc2	T– > C	Ile– > Val	0.58	0	0	0	0	0	0
RNA2	2784	NSvc2	A– > G	Ile– > Thr	0.68	0.16	0.12	0.12	0	0	0
RNA3	104	NS3	T– > G	Phe– > Leu	0.31	0	0	0	0	0	0
RNA3	227	NS3	T– > G	Ile– > Met	0.32	0	0	0	0	0	0
RNA3	485	NS3	T– > A	Phe– > Leu	0	0.17	0.18	0.18	0	0	0
RNA3	644	NS3	G– > A	Lys	0.19	0	0	0	0	0	0
RNA3	661	NS3	T– > C	Asp– > Ala	0	0.2	0.21	0.21	0	0	0
RNA3	1197	IR	A– > G		0.37	0	0	0	0	0	0
RNA3	1226	IR	T– > A		0.36	0.21	0.23	0.18	0	0	0
RNA3	1362	IR	A– > G		0.37	0.22	0.24	0.21	0	0	0
RNA3	2189	CP	G– > A	Asp	0.54	0	0	0	0	0	0
RNA4	216	SP	A– > T	Ile	0.18	0	0	0	0	0	0
RNA4	340	SP	A– > G	Asn– > Asp	0.85	0.22	0.21	0.21	0	0	0
RNA4	526	SP	A– > G	Lys– > Glu	0.85	0	0	0	0	0	0
RNA4	628	IR	G– > T		0	0.6	0.6	0.59	1	1	1
RNA4	721	IR	T– > C		0.47	0.21	0.20[a]	0.19	0.3	0.28	0.39
RNA4	755	IR	T– > A		0.18	0	0	0	0	0	0
RNA4	767	IR	C– > T		0.83	0	0	0	0	0	0
RNA4	940	IR	G– > A		0.85	0.23	0.2	0.21	0	0	0
RNA4	1069	IR	C– > A		0	0	0	0	0.22	0.28	0.29
RNA4	1137	IR	C– > T		0.82	0.16	0.16	0.16	0	0	0
RNA4	1152	IR	A– > T		0	0.71	0.7	0.71	1	1	1
RNA4	1198	IR	G– > T		0	0.68	0.69	0.69	1	1	1

In5d, small brown planthoppers acquiring RSV for 5 d, one replicate; InQ, viruliferous planthoppers, three replicates; P, RSV-infected rice leaves with typical stripe symptom, three replicates.
IR, intergenic region; 5′-NTR, 5′-non-translated region.
[a] , this SNP site was only identified by GATK, not by bcftools. So this site was not counted as a SNP site in InQ samples.

quantitative real-time PCR (qRT-PCR) in planthoppers after injection of the recombinantly expressed SP proteins for 24 h and 48 h. Fragments ranging from 100 bp to 200 bp were amplified from the 19 genes using the corresponding primer pairs (Supplemental Table 1). qRT-PCR was carried out in 20 μL of reaction agent composed of 1 μL of template cDNA, 10 μL of 2 × SYBR Green PCR Master Mix (Fermentas, Waltham, MA, USA), and 0.25 μM each primer on a Light Cycler 480 II (Roche, Basel, Switzerland). The thermal cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 68 °C for 40 s. The transcript level of the planthopper translation elongation factor 2 (*EF2*, evm.model. Contig0.299 in *L. striatellus* genome dataset, (Zhu et al., 2017)) was quantified with the primer pair *EF2*-F/*EF2*-R to normalize the cDNA templates (Supplemental Table 1). The relative transcript level of each gene compared to *EF2* was reported as the mean ± SE. Differences were statistically evaluated using one-way ANOVA followed by a Tukey's test in SPSS 17.0.

3. Results

3.1. Genetic diversity of RSV in planthoppers and in rice plants

To evaluate the population genetic diversity of RSV in the vector insect and host plant, we analyzed SNPs of the same RSV population when it circulates between planthoppers and rice plants. In viruliferous planthoppers, 22, 17, and 21 SNP sites were obtained from individual replicates, and 13 SNPs appeared in the three replicates (Fig. 1A). In RSV-infected rice samples, 6, 8, and 7 SNP sites were identified from individual replicates, and 5 SNPs existed in all three replicates (Fig. 1B). The 13 SNPs in the viruliferous planthoppers did not overlap with the 5 SNPs in the RSV-infected rice (Fig. 1C). In addition, when RSV incubated in the small brown planthoppers for 5 d, 33 SNP sites were found with only one replicate (Table 1); among these SNPs, 6 also appeared in the viruliferous planthopper samples (Fig. 1C).

The SNP sites were plotted along the four RNA segments of RSV.

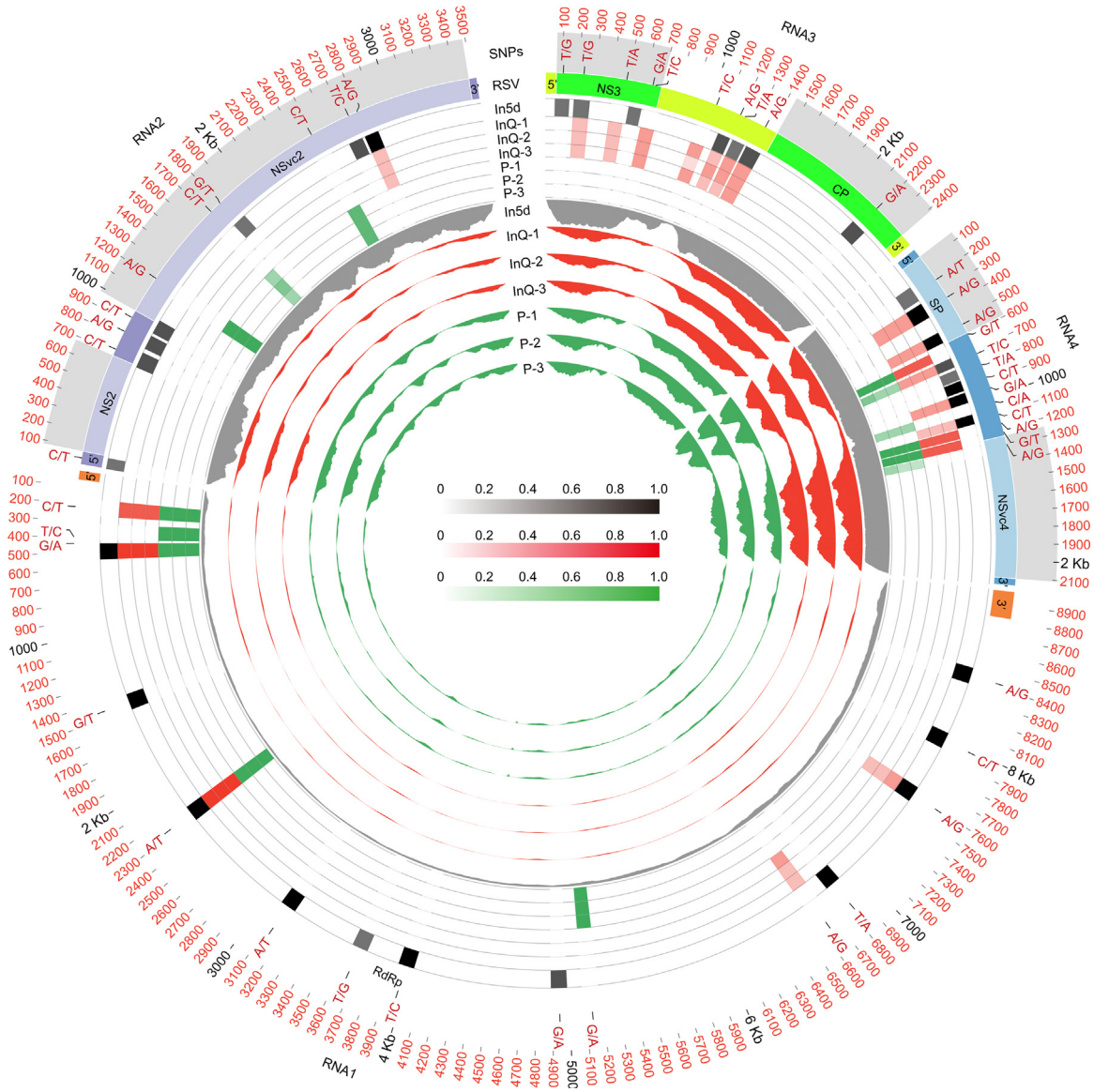

Fig. 2. Circular representation of RSV single nucleotide polymorphism (SNP) distribution and reads coverage along the viral genome in different samples. The outer scale marks genomic positions. The first circle indicates the position and alleles of SNPs. The second circle shows the genome annotation of RSV. Heatmaps on tracks 3–9 represent the ratios of SNP alleles. The heatmap in light gray to black is for SNPs in the small brown planthoppers 5 d after acquiring RSV (In5d), light red to dark red for SNPs in the three replicates of viruliferous small brown planthoppers (InQ), and light green to dark green for SNPs in the three replicates of RSV-infected rice leaves with obvious stripe symptom (P). Plots on tracks 10–16 indicate the reads coverage at different genomic positions.

Among the 33 SNPs detected in RSV incubated in the small brown planthoppers for 5 d, 11 sites were located in RNA1 (1.22 SNPs per kb), 7 sites in RNA2 (1.98 SNPs per kb), 7 sites in RNA3 (2.81 SNPs per kb), and 8 sites in RNA4 (3.72 SNPs per kb) (Fig. 2, Table 1). In viruliferous planthoppers, 2 SNP sites were located in RNA1 (0.22 SNPs per kb), 1 site in RNA2 (0.28 SNPs per kb), 4 sites in RNA3 (1.61 SNPs per kb), and 6 sites in RNA4 (2.79 SNPs per kb) (Fig. 2, Table 1). Therefore, RNA3 and RNA4 possessed a denser SNP distribution than RNA1 and RNA2 when RSV resided in the vector insect. On the other hand, in RSV-infected rice plants, 3 SNPs were located in RNA2 (0.85 SNPs per kb), and 2 SNPs in RNA4 (0.93 SNPs per kb) (Fig. 2, Table 1).

Next, we examined whether SNPs had effects on coding proteins. In RSV incubated in the small brown planthoppers for 5 d, 23 SNPs were located in gene ORFs, including 11 in *RdRp*, 5 in *NSvc2*, 3 in *NS3*, 1 in *CP*, and 3 in *SP*, and 13 SNPs caused nonsynonymous substitutions (Table 1). The other 10 SNPs were located in noncoding regions, including one site in a 5′-untranslated terminal region (NTR) on RNA2, and 9 in the intergenic regions (IR) from RNA2 to RNA4 (Table 1). In viruliferous planthoppers, 6 SNPs were located in the ORFs of *RdRp*, *NSvc2*, *NS3*, and *SP*, and produced 5 nonsynonymous substitutions. The other 7 SNPs were distributed in the IRs of RNA3 and RNA4 (Table 1). In the RSV-infected rice leaves, 3 SNPs were located in the *NSvc2* ORF and produced 2 nonsynonymous substitutions. The other 2 SNPs were located in the IR of RNA4 (Table 1).

Notably, some SNPs that induced nonsynonymous substitutions showed largely divergent substitution ratios among different samples. For example, the substitution ratios at positions 440 and 2274 of RNA1 in the RdRp coding region and positions 340 and 526 of RNA4 in the *SP* coding region were over 0.8 in samples from RSV incubated in the small brown planthoppers for 5 d, while the ratios were no higher than 0.22 in viruliferous planthoppers or in the RSV-infected rice (Table 1). Two sites at positions 399 and 5137 of RNA1 in the *RdRp* coding region had no SNPs within the samples but used completely different nucleotides in the RSV-infected insects and RSV-infected plants (Table 1).

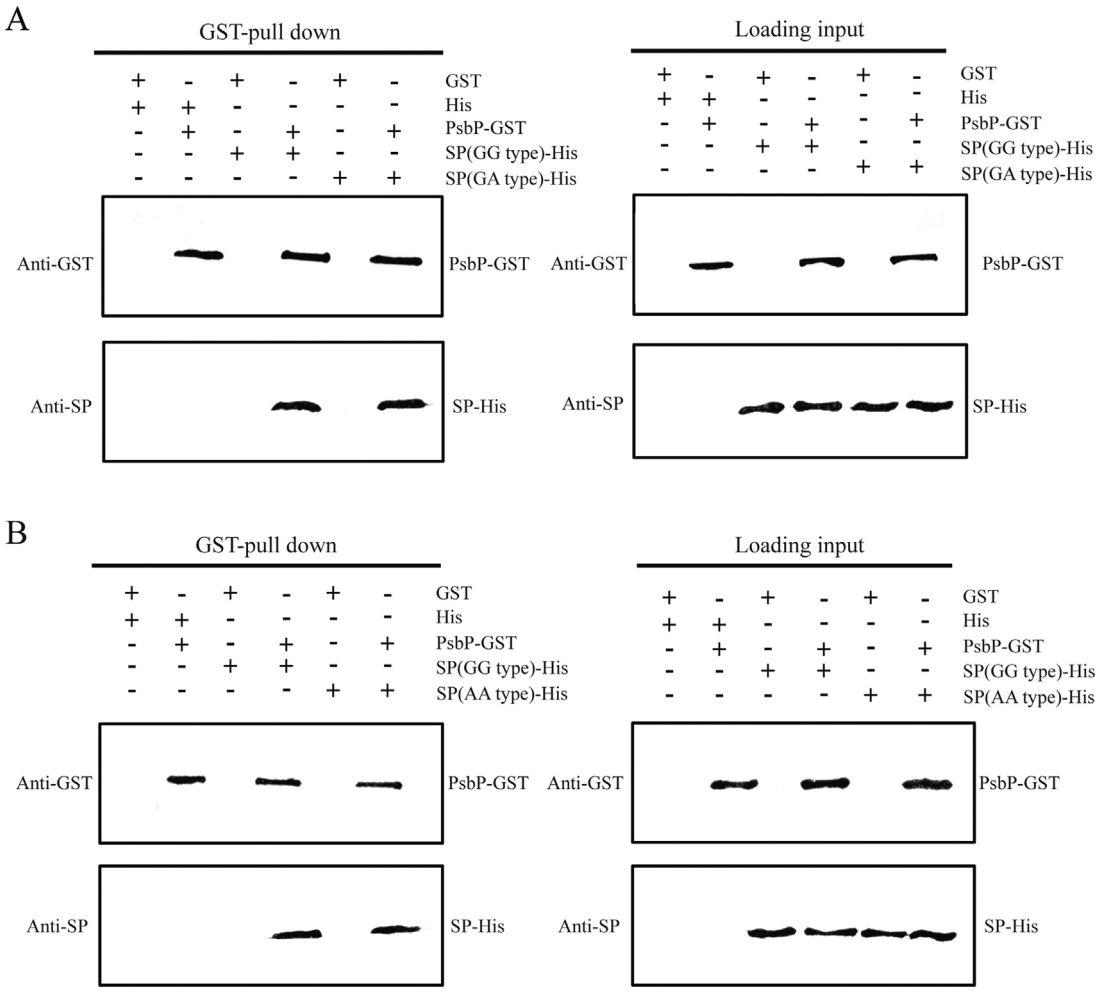

Fig. 3. Interactions of the three SP genotypes with rice PsbP in a GST-tag pull-down assay. (A) Interactions of the SP GG type or GA type with PsbP. (B) Interactions of the SP GG type or AA type with PsbP. An anti-SP monoclonal antibody and an anti-GST polyclonal antibody were used to recognize the target proteins. Expression products from the pET28a vector (His) and pGEX-3X vector (GST) were used as negative controls.

3.2. Existence of three genotypes of SP gene

SP protein is strongly correlated with RSV disease symptom development in rice (Kong et al., 2014; Zhu et al., 1992) and plays a critical role in viral spreading in planthoppers (Wu et al., 2014). Based on the transcriptome data, 85% of the nucleotides at positions 340 or 526 of RNA4 were guanines (G) and 15% were adenines (A) in the planthoppers 5 d after acquiring RSV. This led to nonsynonymous substitutions at the 96th codon (Asn/Asp) and the 158th codon (Lys/Glu) of SP (Table 1). In contrast, only approximately 20% of nucleotides at position 340 were G, and no G were observed at position 526 in the viruliferous planthoppers. In RSV-infected rice, these two positions did not show SNP (Table 1). Thus, the GG genotype of *SP* at positions 340 and 526 may mainly exist in the early infection of RSV in the planthoppers and be gradually substituted by the other two genotypes during viral transmission. To verify these genotypes, the full-length of *SP* from the planthoppers 5 d after acquiring RSV, the viruliferous planthoppers, and the RSV-infected rice was cloned and sequenced. Three genotypes of *SP*, including GG, AA and GA, were detected in the planthoppers 5 d after acquiring RSV, while only the AA and GA genotypes were detected in the viruliferous planthoppers and RSV-infected rice.

3.3. Interactions of three genotypes of SP with RSV CP or rice PsbP

Nonsynonymous SNPs can affect the protein-protein interactions (Zhao et al., 2014). In rice, RSV SP disturbs the integrity of grana stacks in the chloroplast through binding with PsbP, a photosystem II subunit (Kong et al., 2014; Zhao et al., 2016b). In planthoppers, SP facilitates RSV virion spreading by interacting with RSV CP (Wu et al., 2014). We analyzed the interactions of three genotypes of SP with PsbP or CP using GST-tag or His-tag pull-down assays to determine whether these SNPs affected the protein-protein interactions. The results showed that all of the three genotypes of SP-His recombinant proteins were able to interact with the PsbP-GST or CP-GST recombinant protein, and the binding capacity was similar for the three genotypes (Figs. 3 and 4). Therefore, the two SNPs at positions 340 and 526 of RNA4 did not affect the interactions of SP with RSV CP or with rice PsbP, indicating that the three genotypes of SP may have no differences in inducing disease symptom development in rice and virion spreading in planthoppers.

3.4. Immune response of planthoppers to the three genotypes of SP

Recombinant proteins encoded by the three SP genotypes were in vitro-expressed and purified before microinjection into the hemolymph of nonviruliferous planthoppers. The transcript levels of 19 immune-related genes involved in the Toll pathway, reactive oxygen functions, pattern recognition molecules and immune-responsive effector pathways of planthoppers were evaluated and compared among the three SP-injection groups and the mock group using qRT-PCR at 24 or 48 h after injection (Supplemental Table 2). These genes were differentially expressed in the salivary glands or guts of viruliferous planthoppers

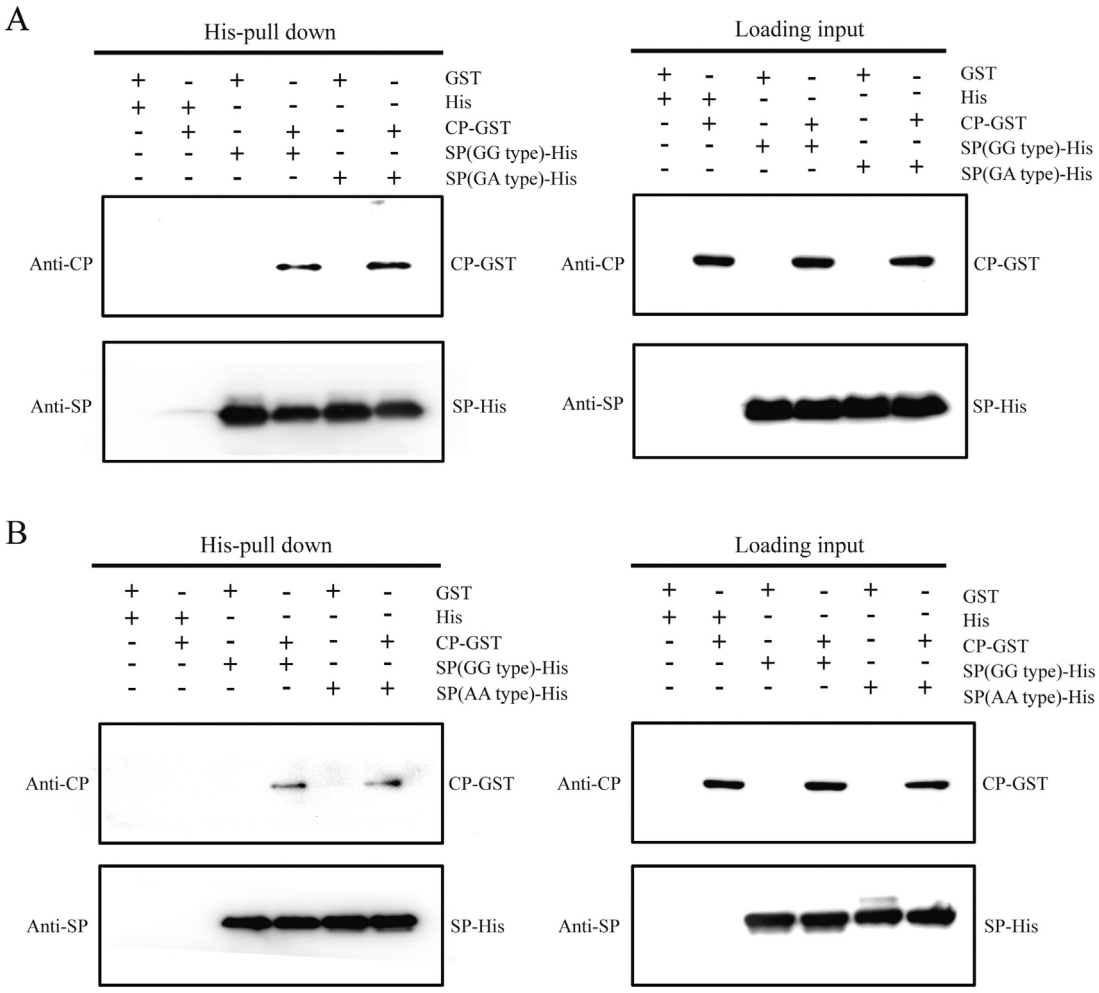

Fig. 4. Interactions of the three SP genotypes with RSV CP in a His-tag pull-down assay. (A) Interaction of the SP GG type or GA type with CP. (B) Interactions of the SP GG type or AA type with CP. An anti-SP and an anti-CP monoclonal antibody were used to recognize the target proteins. Expression products from the pET28a vector (His) and pGEX-3X vector (GST) were used as negative controls.

compared to the organs of nonviruliferous planthoppers (Zhao et al., 2016a).

The qRT-PCR results showed that the GG genotype of SP significantly upregulated the transcript levels of the *Niemann-pick type C-2* and *GRP7* gene in the pattern recognition molecule pathway at 48 h, while the GA or AA genotype of SP did not affect these transcripts compared to the mock group (Fig. 5). Other genes in this pathway, *GRP4* and *PGRP-LC*, did not respond to the three genotypes of SP (Fig. 5). For the immune-responsive effector pathway genes, the GG genotype of SP remarkably upregulated the expression of *defensin B*, *nitric oxide synthase*, and *fibrillin* at 48 h, while the GA or AA genotype of SP did not (Fig. 6). The expression of *defensin B* was activated by all three types of SP at 24 h, and the GG genotype had the largest effect (Fig. 6). Other genes in this pathway, such as *prophenoloxidase*, *c-type lysozyme*, and *CD109-antigen*, did not respond to the three types of SP (Fig. 6). Four Toll pathway immune genes (encoding a proclotting enzyme-3, a serine protease-like precursor, a serine protease snake-3, and a trypsin) and five reactive oxygen function immune genes (encoding a peroxidase, two peroxiredoxins, a superoxide dismutase, and a mitochondrial manganese superoxide dismutase) showed similar expression levels before and after injection of each of the three types of SP, indicating that these two immune pathways possibly did not respond to the three genotypes of SP (Supplemental Fig. 1 and Supplemental Fig. 2). These results indicated that the GG genotype of SP induced a stronger immune response than the other two genotypes in planthoppers.

4. Discussion

Here, we reported the variations of the same population of RSV during the circulation between vector planthoppers and host rice from the view of SNPs. Although more RSV SNP sites were observed in viruliferous planthoppers (13 SNPs) than in rice with disease symptoms (5 SNPs), we did not confirm whether a bottleneck occurred and impacted on SNP diversity in this study due to lacking continuous samplings during viral acquisition, incubation, and transmission in the vector insect and host plant. A relative study was ever carried out with the horizontal transmission of artificial populations of *Cucumber mosaic virus* (CMV) from aphid vectors to host plants (Ali et al., 2006). All 12 viral mutant strains were detected in plants after mechanical inoculation, while only three of the 12 mutants were present in the plants after transmission by aphid vectors, suggesting a bottleneck during the horizontal vector transmission of CMV. We speculate that the reduced variation in RSV in rice may be related to the strong immune response of rice such as the upregulated expressions of peroxidase biosynthesis genes, leucine-rich repeat receptor-like protein kinase genes, and pathogenesis-related protein genes (Hao et al., 2011; Zheng et al., 2013). On the other hand, RSV minimizes the effect on the immune system of its vector insect; for example, RSV does not provoke the JAK/STAT and Imd pathways (Zhang et al., 2010; Zhao et al., 2016a).

The GG type of SP was detrimental for RSV residing in planthoppers. The GG type of SP induced a stronger immune response than the other two genotypes in planthoppers, as shown by its upregulation of several

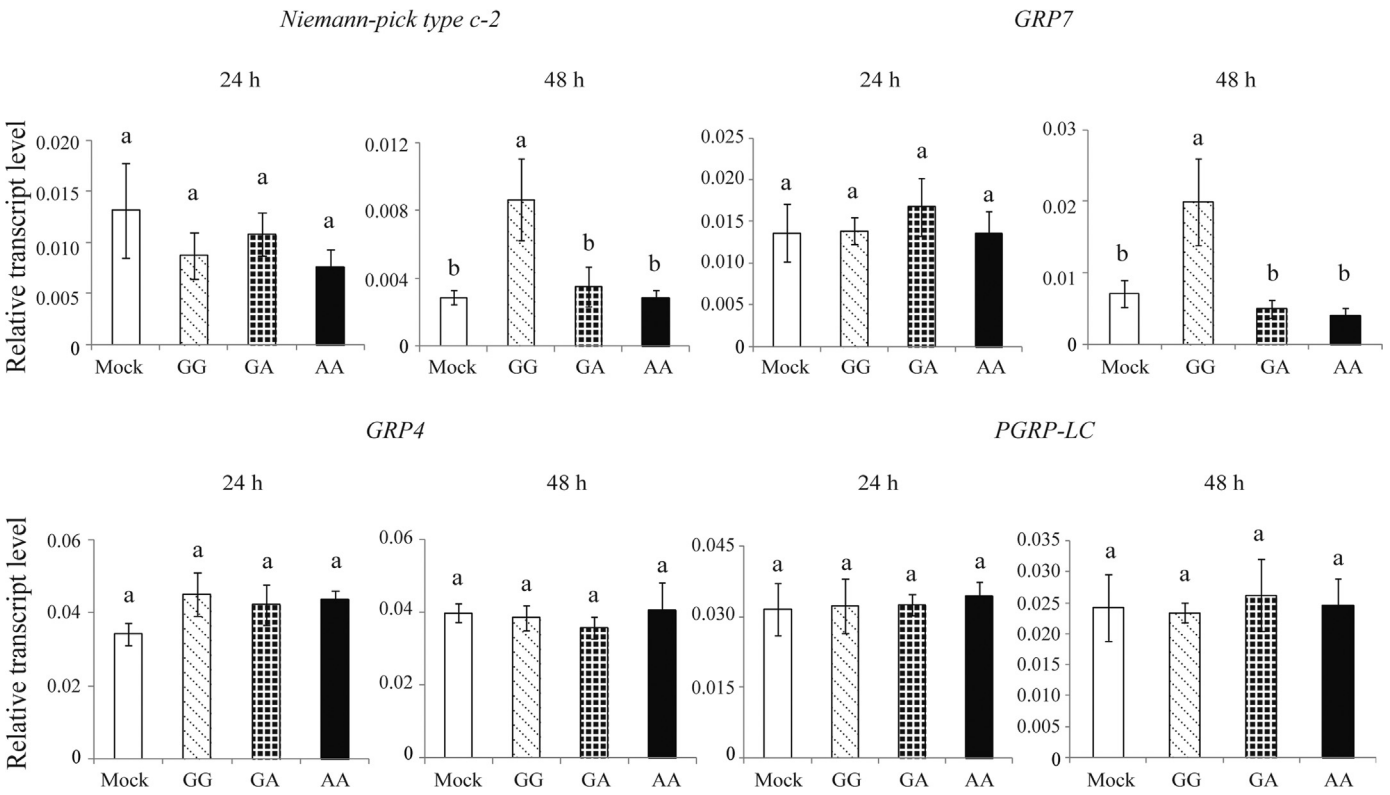

Fig. 5. Immune response of the pattern recognition molecule pathway in small brown planthoppers to the three SP genotypes. The relative transcript levels of 4 pattern recognition molecule genes normalized to the translation elongation factor 2 were evaluated using quantitative real-time PCR after 24 or 48 h of injection with the purified GG, GA, or AA type of SP recombinant proteins. The insects injected with equal volumes of the purified products from the pET28a vector were used as mock group. Six to eight biological replicates with five insects per replicate were prepared. The data were reported as the mean ± SE. Differences were statistically evaluated using one-way ANOVA followed by a Tukey's test in SPSS 17.0. Different letters indicate significant differences.

genes encoding pattern recognition molecules and immune-responsive effectors. This explained the elimination of the GG type during vector transmission of RSV. Maintaining diverse quasispecies may help RNA virus populations to adapt to novel environmental conditions, but it also enable accumulation of deleterious mutations (Holland and Domingo, 1998). There are two main forces that reduce genetic variation of RNA virus populations during the transmission cycle. One is genetic drift that decreases genetic variation in a random way (Li and Roossinck, 2004). The other is negative selection which specifically limits genetic variation (Lequime et al., 2016). The stronger immune reaction induced by the GG type of SP indicates that SP may experience a negative selection rather than a genetic drift, which increases viral fitness during the horizontal transmission. Strong negative selection has been reported on the seven genes of different RSV isolates in rice (Huang et al., 2013; Wei et al., 2009). As many as 341 viral SNP sites have been detected in RSV-infected rice plants collected from fields (Huang et al., 2015), which is much more than the number we observed in the lab-cultured isolate in this study. Genetic drift and negative selection decreased the genetic diversity of the isolate during the 10-year maintenance.

SNP sites of RSV were more frequently distributed to *RdRp*, *NSvc2*, and the intergenic region of RNA4 than other regions. These SNPs might also contribute to viral fitness in hosts. RdRp is responsible for RNA replication and gene transcription. Variation in the RdRp sequence could influence its enzymatic activity. NSvc2 is a putative membrane glycoprotein because it is similar to membrane glycoproteins of viruses in the family *Bunyaviridae* (Zhao et al., 2012). However, there has been no evidence demonstrating that RSV is an enveloped virus. The variations in the intergenic region of RNA4 could affect the RNA structure or virus-derived small interfering RNAs (Yang et al., 2018; Xu et al., 2012). The genetic variation of several arboviruses, such as the Zika virus (Wang et al., 2016), dengue virus (Filomatori et al., 2017; Lequime et al., 2016; October et al., 2015; Sim and Hibberd, 2016), and influenza viruses (Illingworth et al., 2014; Van den Hoecke et al., 2015), has been reported during their spreading between invertebrate vectors and vertebrate hosts. Significant changes in viral untranslated regions, RNA structures, coding RNA sequences, and protein sequences or structures were found during cross-host transmission. Using the Zika virus as an example, nearly all of the human strains belonged to the Asian lineage, while eight mosquito strains belonged to the African lineage. Six amino acid substitutions (I110V, K143E, A148P, V153M, H157Y, and V158I) in the precursor region of the premembrane protein, one of the structure proteins of the Zika virus, were found between the African and Asian strains, resulting in a dramatic predicted structural change of the premembrane protein. One mutation, A148P, which was only detected in human isolates, could possibly play a critical role in maintaining human infectivity (Wang et al., 2016). In contrast, we only detected one SNP site in the structural protein CP of RSV, and this SNP did not lead to an amino acid change.

CRediT authorship contribution statement

Wan Zhao: Investigation, Writing-original draft. **Qianshuo Wang:** Investigation, Writing-original draft. **Zhongtian Xu:** Software, Data curation. **Renyi Liu:** Writing-review & editing. **Feng Cui:** Writing-review & editing.

Acknowledgements

This work was funded by the National Key R&D Program of China (No. 2017YFD0200400), the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDB11040200) and the National

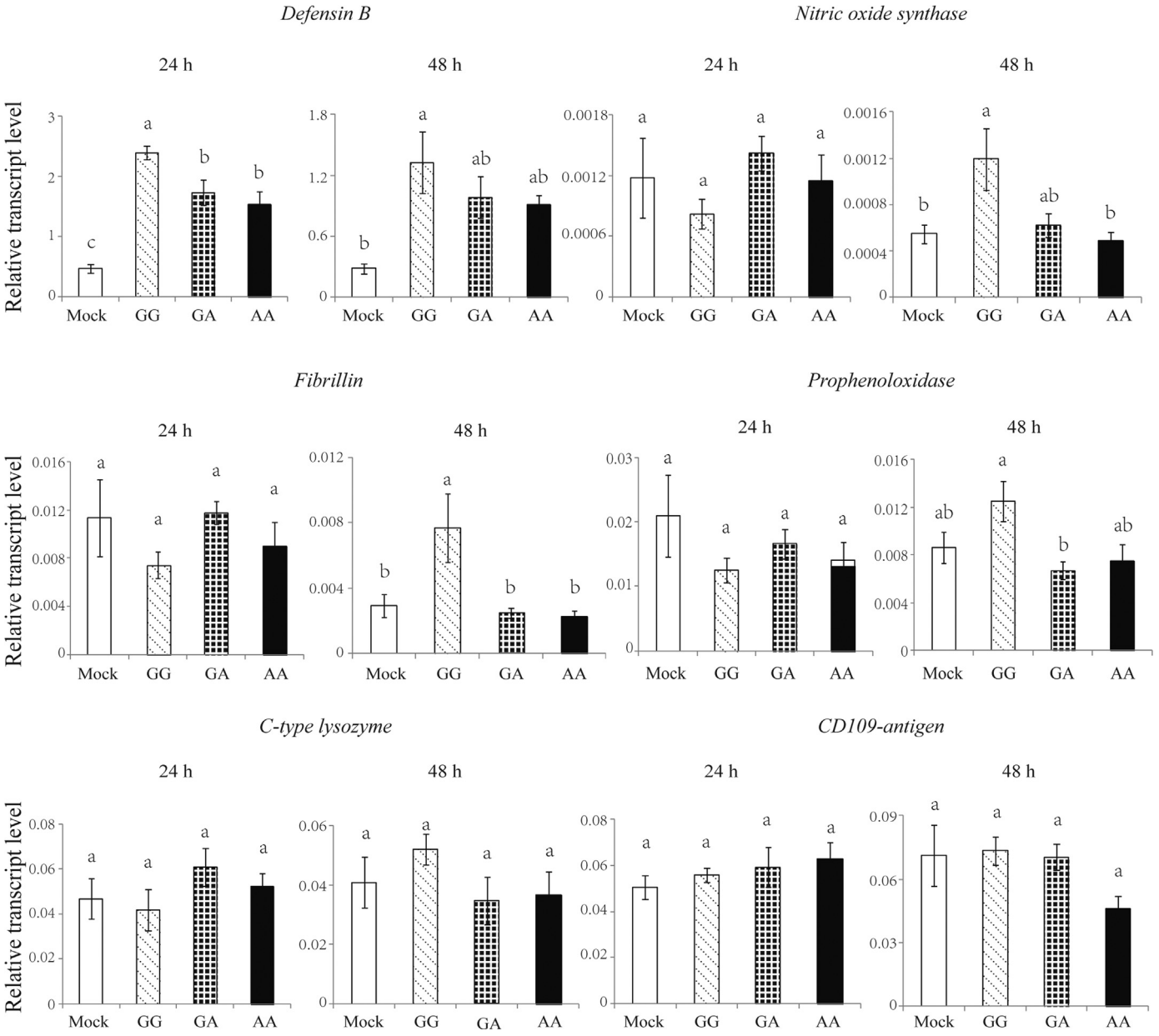

Fig. 6. Immune response of the immune-responsive effector pathway in small brown planthoppers to three genotypes of SP. The relative transcript levels of 6 immune-responsive effector genes normalized to the translation elongation factor 2 were evaluated using quantitative real-time PCR after 24 or 48 h of injection with purified the GG, GA, or AA type of SP recombinant proteins. The insects injected with an equal volume of the purified products from the pET28a vector were used as the mock group. Six to eight biological replicates with five insects per replicate were prepared. The data were reported as the mean ± SE. Differences were statistically evaluated using one-way ANOVA followed by a Tukey's test in SPSS 17.0. Different letters indicate significant differences.

Natural Science Foundation of China (No. 31802017).

Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.11.011.

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