RNAi silencing of rice black-streaked dwarf virus P10 and two insect vector genes to reduce virus transmission protects rice plants against RBSDV

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ABSTRACT

Rice black-streaked dwarf virus (RBSDV) is a virus transmitted by Laodelphax striatellus (L. striatellus), which poses a serious threat to rice production. P10, a major outer capsid protein of RBSDV, which plays a key role in viral infection. In this study, two L. striatellus transmission-related proteins, novel cuticular protein (NCuP) and receptor for activated protein kinase C (RACK), were identified as interacting with P10. Results demonstrated that virus-induced NCuP and RACK were silenced using double-stranded RNA (dsRNA) to reduce the quantity of virus in L. striatellus. The detection results after inoculation of RBSDV by L. striatellus showed that rice plants sprayed with dsP10 +NCuP and dsP10+RACK and transgenic rice plants expressing both had higher disease resistance than those expressing dsP10, dsNCuP and dsRACK alone. Therefore, simultaneously interfering with both the expression of viral protein and the proteins that interact with them from insect vectors could enhance the control of viral transmission.

Introduction

Viral infection causes enormous losses in crop productivity and crop quality, imparting a plethora of threat to food security (Zorzatto et al. 2015; Bian et al. 2020). These losses are mainly attributed to virus-induced abnormal growth and development, as exhibited by virus-induced disease symptoms in plants (Lian et al. 2016). Most plant viruses are transmitted among plants by insect vectors, especially insects with piercing sucking mouthparts, such as aphids, whiteflies, leafhoppers, planthoppers, and thrips (Hohn 2007). Insect vectors transmit plant viruses in two ways: circulative transmission and non-circulative transmission. Circulative viruses are categorized into propagative and non-propagative (Hogenhout et al. 2008). The non-propagative viruses (e.g., luteiviruses) could exist in the host for a long time but could not be propagated in the host. The propagative viruses need to move and replicate in insect tissues, including moving through the alimentary canal of the insect, crossing the gut epithelial cells; and then being released into the haemolymph to spread to other tissues and organs. The virus ultimately infects the salivary glands and crosses the epithelial barriers to transfer to new host plants during feeding through the salivary released (Schwarz et al. 2014). Therefore, the success and efficiency of viral transmission in insects require specific interactions between the virus and the insect vectors.

The specific interaction between plant viruses and their-transmitting vectors involves many viral receptors and proteins (Zhao et al. 2019). Rice black stripe dwarf virus (RBSDV) is a member of the genus Figiviruses; it is transmitted by L. striatellus in a persistent circulative pattern (Yin et al. 2013). Several insect proteins interact with RBSDV-encoded proteins and may be linked to successful transmission. Zhou (2013) screened an L. striatellus cDNA library with RBSDV P10 as bait protein and identified 14 proteins, including novel cuticular protein (NCuP); receptor for activated protein kinase C (RACK) and actin1. However, little is known about the physiological roles of these interaction proteins in the viral transmission in L. striatellus, some of these proteins function in many important physiological activities, such as cell movement, endocytosis and morphological maintenance and growth (Seddas et al. 2004; Ohkawa and Welch 2018). Therefore, it is of great theoretical significance and application value to study the role of these proteins in the viral transmission mechanism of insect vectors.

RNA silencing is a sequence-specific gene-silencing mechanism triggered by double-stranded RNA (dsRNA); it is thought to be an inherent defence mechanism in eukaryotes in terms of preventing viral replication and protecting cells from viral invasion (Csorba et al. 2009; Sidahmed and Wilkie 2010; Zhang et al. 2017a). Several strategies have been developed in the past two decades on the basis of interference with the expression of viral genes or insect vector genes (Jian and Du 2018; Shi et al. 2019). For example, using dsRNA produced by an in-vitro expression system ingested into insects to silence the genes of insect vectors or virus could efficiently interfere with viral infection (Shi et al. 2019). In addition, transgenic plants could be engineered to express a single hairpin dsRNA targeting virus-binding protein genes from insects or virus-related protein genes from virus to enhance their resistance to viruses (Zaidi et al. 2016; Jafarzade et al. 2019). Many strategies to engineer RNA interference (RNAi)-mediated plant viral resistance have met with varying degrees of success, including attenuated and delayed symptoms, immunity and absence of resistance (Mansoor et al. 2006; Guo et al. 2018). However, the strategies for RNAi-mediated disease
resistance are relatively simple and inefficient at present, and most of them involve unilateral interference with the expression of viral genes or insect vector genes to control viral transmission. Therefore, obtaining an improved antiviral effect through bidirectionally blocking the virus-host-vector interactions is possible by simultaneously interfering with the expression of viral genes and insect vector genes.

The genome of RBSDV contains 10 linear dsRNAs (S1–S10) and encodes 13 proteins (Azuhata et al. 1993; Wang et al. 2003). In these RNA segments, S10 has one open reading frame and encodes P10, a major outer capsid protein. Several studies have indicated that besides playing an important role in the formation of RBSDV particles, P10 interacts with the insect vector or host proteins to regulate viral replication and transmission (Liu et al. 2007; Lu et al. 2019). In the present study, yeast-two-hybrid (Y2H) screening against an L. striatellus cDNA library was performed, and two host targets of P10, NCuP and RACK, were identified. NCuP and RACK are thought to be involved in viral transmission and intercellular transport of the virus, respectively (Shuo et al. 2011). The effect of interfering with the expression of RBSDV P10 and the two transmission-related genes of the virus in L. striatellus were analysed by feeding with artificially synthesized dsRNAs in vitro on RBSDV transmission. Then, the dsRNAs of NCuP, RACK, P10+NCuP and P10+RACK were expressed using a bacterial prokaryotic expression system, and the effect of viral resistance after spraying these dsRNAs on rice was determined. In addition, transgenic rice carrying NCuP-RNAi, RACK-RNAi, P10+NCuP-RNAi and P10+RACK-RNAi expression vectors was generated, and viruliferous (RBSDV) L. striatellus was fed on these plants to analyze the resistance of transgenic rice to RBSDV. The results demonstrated that simultaneously expressing the dsRNA of viral protein genes and that of its interaction protein gene from the insect vectors is an effective method to prevent viral transmission. This study elucidated the importance of identifying the appropriate viral target genes and virus transmission-related protein genes of the insect vector for RNAi to effectively inhibit RBSDV infection in rice.

Materials and methods

Plant and insect material

Non-viruliferous and viruliferous L. striatellus were obtained from rice fields and the laboratory of Zhou Yijun, a professor at the Jiangsu Academy of Agricultural Sciences, and reared on 2–3 month old rice plants Lindao10 under controlled environmental conditions (25°C, 16 h light/8 h dark). Insects were transferred to fresh seedlings every week. WT Lindao10, which was grown in the field at the experimental stations of Shandong Agriculture University, was used to generate transgenic lines. The number of surviving insects settling on each transgenic plant and WT plant was counted once two day for 12 days.

Yeast two-hybrid (Y2H) assay

The full-length coding regions of P10, NCuP and RACK were cloned into pGADT7 or pGBK7 vectors. Yeast transformation and screening were performed in accordance with the manufacturer’s instructions (Clontech, Mountain View, CA, USA). Saccharomyces cerevisiae AH109 cells were co-transformed with specific bait and prey constructs and transferred on SD medium lacking tryptophan/leucine (SD-L-W) (for transformation control) and tryptophan/leucine/adenine (SD-L-W-H-Ade) (for selection) media supplemented with X-a-Gal and Aureobasidin A (100 ng/mL).

Firefly luciferase complementation imaging (LCI) assays

The full-length coding regions of P10, NCuP and RACK were cloned into pCAMBIA1300-NLuc or pCAMBIA1300-cLuc vectors. LCI assay was performed as previously described, and all of the related constructs were transformed into Agrobacterium tumefaciens (A. tumefaciens) strain GV3101 (Yang et al. 2017). Specific combinations of A. tumefaciens were infiltrated into different positions in the same leaves of Nicotiana benthamiana for 60 h. Five minutes before detection, 0.2 mM luciferin (Promega, Madison, WI, USA) was uniformly infiltrated into the same positions as A. tumefaciens. Subsequently, luciferase activity was measured using a low-light cooled CCD imaging apparatus (Lumina II, Waltham, MA, USA).

Protein pull-down assays

In vitro pull-down assays were carried out as previously described (Yu et al. 2019). Full-length coding regions of P10, NCuP and RACK genes were cloned into pGEX-4T-3 or pET30a vectors, respectively. The recombinant protein P10-GST, NCuP-His and RACK-His were produced in E. coli Rosetta strain (Transgene; Beijing, China), and purified using a GST protein purification kit (CWBO; Beijing, China) and 6× HIS-Tagged Protein Purification Kit (CWBO) according to the manuals, respectively. The purified P10-GST or GST proteins were incubated with GST beads in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4) at 4°C for 60 h. Five minutes before detection, 0.2 mM luciferin (Promega, Madison, WI, USA) was uniformly infiltrated into the same positions as A. tumefaciens. Subsequently, luciferase activity was measured using a low-light cooled CCD imaging apparatus (Lumina II, Waltham, MA, USA).

Preparation of insect samples, RNA extraction and qRT-PCR

Non-viruliferous L. striatellus at different developmental stages were collected. 1st: first-instar; 2nd: second-instar; 3rd: third-instar; 4th: fourth-instar; 5th: fifth-instar. Approximately 60 individual insects at a specific developmental stage were collected for the assay. Viruliferous (RBSDV) L. striatellus was prepared by allowing second instar L. striatellus nymphs to feed on RBSDV infected rice seedlings for 2 days. The insects were then allowed to feed on healthy rice seedlings for 1–4 days and collected, respectively. Insects at the same developmental stage reared on healthy rice seedlings were used as controls. RNA was extracted with TRIzol Reagent (Invitrogen) in accordance with the manufacturer’s instructions (Clontech, Mountain View, CA, USA). Saccharomyces cerevisiae AH109 cells were co-transformed with specific bait and prey constructs and transferred on SD medium lacking tryptophan/leucine (SD-L-W) (for transformation control) and tryptophan/leucine/adenine (SD-L-W-H-Ade) (for selection) media supplemented with X-a-Gal and Aureobasidin A (100 ng/mL).
with the manufacturer's instructions. A concentration of total RNA (2 μg) was reverse transcribed using the Fast Quant RT kit (Tiangen) with oligo-(dT) primers in accordance with the manufacturer's instructions. qRT-PCR reactions were performed using the SYBR Green Real-Time PCR Master Mix (Vazyme) following the manufacturer's instructions. Relative transcript levels were calculated using the 2^(-ΔΔCt) method. Actin was used as a reference gene. Each data-set was derived from three biological repeats. The primers are listed in Table S1.

Production of dsRNA via in-vitro expression system

For dsRNA synthesis, the full-length sequences of NCuP, RACK, P10 and GFP were amplified via PCR using gene-specific primers with 23 bases of the T7 RNA polymerase promoter added in the primers in accordance with the protocol of the T7 RiboMAX Express RNAi System (Promega). The PCR products were then used as templates for dsRNA synthesis on the same system. After synthesis, the dsRNA was purified via isopropanol precipitation. Agarose gel electrophoresis was used to assess its purity and integrity. The primers are listed in Table S1.

Preparation of dsRNA by bacterial prokaryotic expression

Bacterial prokaryotic expression of dsRNA assay was performed as previously described (Tenllado et al. 2003). NCuP, RACK, P10 and GFP fragments were amplified using specific primers (Table S1) and inserted at inverted repeats into the pGEM-TEasy vector to generate a hairpin RNAi construct. The plasmids were transformed into M-JM109 LacY cells, the recombinant plasmid was induced by IPTG and dsRNA was extracted using TRIzol Reagent (Yin et al. 2009). Then, 1 L cell pellets of M-JM109 LacY/ dsP10, dsNCuP, dsRACK, dsP10+NCuP and dsP10+RACK were pelleted by centrifugation and resuspended in 20 mL of 50 mmol/L Tris and 10 mmol/L EDTA at pH 7.5 (Tenllado et al. 2003). The cell suspension was subjected to sonication (20 kHz, 15 min) and centrifugation at 9000 rpm for 20 min. The supernatant was tested for interference activity on viral infection. The primers are listed in Table S1.

Feeding L. striatellus experiments with dsRNA

For dsRNA synthesized artificially in vitro, the second instar nymphs of non-viruliferous L. striatellus were fed with artificial feed mixed dsRNA (500 ng/μL of dsNCuP and dsRACK, 500 ng/μL of GFP dsRNA as the control). After 2 d of feeding, half of the insects were used to detect the expression levels of target genes by qRT-PCR. After the removal for 12 d, the viral content in each plant was detected using indirect ELISA.

Detection of RBSDV by ELISA

Rice plants were incubated in water for 4 days and RBSDV detected by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Agdia, Elkhart, IN). To evaluate RBSDV infection, pieces (about 1 cm) of leaf sheath plus stem tissue were harvested from ten RBSDV-inoculated rice plants per line and subjected to DAS-ELISA. To quantify RBSDV in rice plants, frozen samples of plants were ground in the Multi-beads shocker® and suspended in 10 volumes of PBS. Each extract was diluted 20-fold with PBS and subjected to DAS-ELISA, as described previously (Baton et al. 2009). The proportion of rice plants infected with RBSDV was calculated and expressed as arcsin(√(percent infection/100)) to normalize data and associated residuals.

Vector construction and plant transformation

For the RNAi vectors of NCuP, RACK and P10, cDNA fragments were amplified using PCR with specific primer pairs (Table S1) and inserted as inverted repeats into the pCAMBIA-1300b vector to generate a hairpin RNAi construct via different restriction enzyme sites. The final RNAi vector was transformed into A. tumefaciens EHA105. Transgenic rice plants were generated by the Agrobacterium-mediated transformation of rice calli and further selected by phosphinothricin (PPT) resistance, and these plants were regenerated from the transformed calli (Ma et al. 2011). The regenerated transgenic rice plants were grown in a greenhouse, and self-fertilized homozygous T2 progeny of the transgenic plants were used for further analysis.

Northern blot analysis of mRNA and siRNA in transgenic plants

Total RNAs were extracted from plant tissues with TRIzol Reagent (Invitrogen, USA). Approximately 20 μg of total RNA was separated on a denaturing 1.5% formaldehyde agarose gel. The formaldehyde gels were transferred into a Hybond N+ membrane (Amer sham) with 20× SSC and hybridized with DIG-labelled RNA probes in accordance with the manufacturer's instructions (DIG Northern Starter Kit, Roche). The siRNA of transgenic plants and their WT (as control) were extracted with PureLink miRNA Isolation Kit (Invitrogen) in accordance with the manufacturer's instructions. The samples were heated treated in siRNA loading buffer, equally loaded onto a 15% polyacrylamide gel containing 7 M of urea; transferred to a Hybond-N+ membrane through electrophoresis and further fixed via UV cross-linking. The following hybridization was the same as the total RNA Northern blot.

RBSDV resistance analysis of transgenic rice plants

The rice seed was germinated in a transparent glass and covered with gauze. For viral infection, rice plants were exposed
to the second to third instar viruliferous nymphs of viruliferous (RBSDV) or non-viruliferous L. striatellus at a ratio of 1:2 (two insects per plant) for 72 h when they were about 2 weeks old. After the seedlings were fed for 3 d, the gauze was removed and insecticide was sprayed to kill the L. striatellus. Then, the seedlings were transplanted to an insect-free greenhouse at 25 ± 3°C under natural sunlight for development and evaluation of symptoms. The incidence rate of RBSDV was calculated after 45 d, and the viral content of rice plants was detected using indirect ELISA (Baton et al. 2009).

There were 50 seedlings of each transgenic line, and the incidence of each line was calculated 45d after inoculation, which was classified into High resistance, Middle resistance, Middle susceptibility, Susceptibility and High susceptibility according to the standard. Finally, the average disease resistance ratio (the ratio of the sum of the middle and high resistance rates to the total number of transgenic lines) of each transgenic plant was calculated and compared.

Standard: High resistance, incidence 0.0% to 5%; Middle resistance, incidence 5.1% to 15%; Middle susceptibility, incidence 15.1% to 30%; Susceptibility, incidence 30.01% to 50%; High susceptibility, incidence >50%. The first two levels are collectively referred to as disease resistance; the last three levels are recorded as susceptibility.

Results

Isolation and analysis of proteins that interact with RBSDV P10 in L. striatellus

A L. striatellus cDNA library was screened using RBSDV P10 (Genbank No. 297433) as the bait through Y2H assay to identify the host factor(s) that interacted with P10 during RBSDV infection. Several positive cDNA clones were identified, and the NcuP (Genbank No. KT724284) and RACK (Genbank No. KT724285.1) of L. striatellus, which are potentially involved in the viral transmission in L. striatellus (Seddas et al. 2004; Cormann 2009), were selected for further study. The results showed that yeast cells co-transformed with pGADT7-P10/pGBK77-NcuP, pGADT7-P10/pGBK77-RACK, pGBK77-P10/pGADT7-NcuP and pGBK77-P10/pGADT7-RACK were able to grow on selective medium (SD-L-W-H-Ade supplemented with X-a-gal, Figure 1a). This finding indicated that specific interactions occurred between the NcuP and RACK of L. striatellus and RBSDV- P10 in the Y2H system. These interactions were further confirmed by firefly luciferase complementation imaging (LCI, Figure 1b). P10 was cloned into the pCAMBIA1300-cLuc (P10-cLuc) vector, whilst NcuP and RACK were cloned into separate pCAMBIA1300-nLuc (NcuP-nLuc and RACK-nLuc, respectively) vectors. Strong LUC activity was observed when P10-cLuc was co-infiltrated with NcuP-nLuc or RACK-nLuc in N. benthamiana leaves but not in leaves transformed with empty vectors (Figure 1b). To this end, we performed a pull-down assays. GST-P10 or GST was precipitated with GST agaro and then incubated with His-NcuP and His-RACK, respectively, and detected with anti-His antibody. Both NcuP and RACK protein were pulled down by GST-P10 but not GST (Figure 1c). These results confirmed that P10 physically interacted with RACK and NcuP.

Analysis of the expression pattern of NcuP and RACK in L. striatellus

The expression levels of NcuP and RACK in non-viruliferous L. striatellus were examined at different developmental stages by quantitative reverse transcription-PCR (qRT-PCR) to further understand their role in L. striatellus. The results showed that these genes were expressed during the whole development process of L. striatellus, and the expression varied for the different developmental stages. The highest expression of NcuP was observed in first and second instar nymphs (Figure 2a), whereas the highest expression of RACK was observed in second instar nymphs (Figure 2b).

The effect of RBSDV infection on the expression levels of NcuP and RACK was also examined. The results showed that RBSDV infection changed the transcription of NcuP and RACK in viruliferous L. striatellus. The expression of NcuP was up-regulated in all ages, and the highest level was found in the first instar, nearly 2.5 times higher than that of the control (Figure 2c). The expression of RACK was up-regulated by nearly 1.5 times in the first instar nymph and down-regulated by 1.25, 1.4 and 2 times at the second, third and fourth instar. These results suggested that the expression levels of NcuP and RACK were obviously induced by RBSDV infection in young nymphs of L. striatellus (Figure 2d).

Effect of silencing NcuP and RACK by producing dsRNA in the in-vitro expression system on viral transmission in L. striatellus

The potential role of NcuP and RACK in insect vector transmission virus was investigated by ingesting artificially synthesized dsNcuP and dsRACK in vitro to feed upon L. striatellus. After 2 d of feeding, half of the insects were used to detect the expression levels of NcuP and RACK via qRT-PCR. The other part were transferred onto healthy rice for 12 days after feeding on viruliferous rice for 3 d to detected viral content in each plant using indirect ELISA. The results showed that the transcription levels of NcuP and RACK were intensively decreased 2 d after the ingestion of dsRNAs. The expression levels of NcuP and RACK were decreased by 85.76% and 80.43% at 2 day, respectively, and the interference effect lasted from 1 d to 12 d (Figure 3a,b). After 12 d, the proportions of rice plants infected with RBSDV were 28.78% and 36.79%, respectively, in rice plants inoculated with L. striatellus that was fed with dsNcuP and dsRACK (Figure 3c). This decrease was significant compared with that in the control group (68.32%). These results suggested that NcuP and RACK played a vital role in viral transmission in L. striatellus.

Inhibition of RBSDV infection by dsRNA preparation using bacterial prokaryotic expression system

To further elucidate the role of the interaction between NcuP and RACK with P10 in virus transmission by L. striatellus, we expressed the dsRNAs of NcuP, RACK, P10+NcuP and P10+RACK using a bacterial prokaryotic expression system, and fed to viruliferous Laodelphax striatellus to silence the target gene expression. To assess whether the target gene expression was silenced, we used
qRT-PCR to analyze the expression of P10, NCuP and RACK. The data showed that the dsRNA which was induced by different prokaryotic expression vectors all can decrease the expression level of the corresponding genes significantly on day 5 after feeding (Figure 4a–c).

Next, we characterized the effect of viral resistance after spraying these dsRNAs on rice. The results showed that the proportion of virus-positive rice plants sprayed with dsP10, dsNCuP, dsRACK, dsP10+NCuP and dsP10+RACK was significantly lower than that sprayed with dsGFP, and the proportion of virus-positive rice plants sprayed with dsP10+NCuP was the lowest (Figure 4d). These results indicated that RBSDV accumulation in the inoculated leaves was inhibited by the dsRNA preparation of P10, NCuP and RACK via bacterial prokaryotic expression system and the inhibitory effect of dsP10+NCuP was more obvious than that of the others.

The inhibitory effects of the crude bacterial extracts against viral infection at different time points after treatment was determined by inoculating the plants with viruliferous L. striatellus 1, 3, 5, 7 or 9 d after spraying their leaves with dsP10+NCuP preparations (1 μg/μL of total nucleic acid), with dsGFP as the control. After removing the L. striatellus for 12 d, ELISA was used to detect the viral content of these plants. The proportion of infected plants in the first five days after inoculation was low (Figure 4e).

**Resistance of dsRNA-transgenic rice plants to RBSDV infection**

Considering that the dsRNA of prokaryotic expression has a relatively short preventive period, we generated P10-RNAi, NCuP-RNAi, RACK-RNAi, P10+NCuP-RNAi and P10+RACK-RNAi transgenic plants in Lindao10 background. A total of 112 independent PPT-tolerant transgenic rice plants, including 25 P10-RNAi, 21 NCuP-RNAi, 22 RACK-RNAi, 24 P10+NCuP-RNAi and 21 P10+RACK-RNAi transgenic plants, were obtained. No significant morphological differences were found amongst these transgenic lines compared with WT plants. The identification of herbicide PPT resistance and PCR detection showed that the target fragment was successfully inserted into the rice genomic DNA. Northern blot analysis showed that the mRNA of P10, NCuP and RACK were transcribed in the transgenic lines (Figure 5a) and some of them were processed to siRNAs in rice plants (Figure 5a). When the T2 transgenic seedlings were 5–6 cm high, the second instar nymphs of viruliferous L. striatellus were inserted into four heads of each plant. After feeding for 5 d, insecticide was sprayed to kill the L. striatellus. Then, the seedlings were transplanted to a pest control greenhouse for planting. The number of infected plants was counted after 45 d, and the viral content in the plants was detected using ELISA. The RBSDV-infected rice plants show stunting, darkening of leaves and waxy white tumors or black-streaked galls along the veins. The statistical results showed that 40.00%, 38.10%, 31.82%, 62.50% and 52.38% of the transgenic plants demonstrated disease resistance in P10-RNAi, NCuP-RNAi, RACK-RNAi, P10+NCuP-RNAi and P10+RACK-RNAi transgenic lines, respectively (Table 1). ELISA was also performed to quantify the accumulation of RBSDV in all tested plants, and the results showed that this accumulation in transgenic lines exhibiting viral resistance was much lower than that in wild-type (WT) plants (Table S2). Therefore, interfering the expression of transmission-related proteins was correlated with less disease symptoms and decreased percentages of infected transgenic plants.
Figure 2. Expression pattern of NCuP and RACK in *Laodelphax striatellus* (L. Striatellus). (a and b) qRT-PCR analysis of the expression levels of NCuP (a) and RACK (b) in non-viruliferous *L. striatellus* at different developmental stages. (c and d) qRT-PCR analysis of the expression levels of NCuP (c) and RACK (d) in viruliferous and non-viruliferous *L. striatellus*. Actin was used as a reference gene. All the nymphs were collected 1 day after molting. Data information: In (a–d), the expression was normalized relative to that of actin. Values are shown as the mean ± SEM from three biological repeats. One-way ANOVA was used for (a) and (b), followed by Tukey’s HSD test. Significant differences are represented by the different letters above each bar (P < 0.05). In (c) and (d), the asterisks indicate a significant difference compared with the non-viruliferous *L. Striatellus* incubated for 1 day (Student’s t-test, *P* < 0.5, **P** < 0.01).

Figure 3. Silencing of *Laodelphax striatellus* NCuP and RACK after feeding with dsRNA via qRT-PCR. (a and b) qRT-PCR analysis of the expression levels of NCuP (a) and RACK (b) in non-viruliferous *L. striatellus* after feeding with dsRNA via in-vitro expression system. Actin was used as an internal reference. Values are shown as the mean ± SEM from three biological repeats. Asterisk indicates significant difference in treatments compared with the control (Student’s t-test, *P* < 0.5, **P** < 0.01, ***P** < 0.001). (c) Inoculation of plants with viruliferous (RBSDV) *L. striatellus* fed with dsNCuP and dsRACK for 3 days. The positive plant rate was scored after removing the *L. striatellus* for 12 days using ELISA. Values are shown as the mean ± SEM from three biological repeats. The asterisks indicate a significant difference compared with the non-viruliferous *L. striatellus*, and n.s. indicates no significant difference from the wild type (Student’s t-test, *P* < 0.5, **P** < 0.01).
Discussion

The replication and movement of the virus in insect vectors require specific interactions between the virus and the components of the insect vector (Hogenhout et al. 2008; Schwarz et al. 2014). The present study focused on RBSDV P10, a coat protein that plays an important role in regulating viral replication and transmission (Sun et al. 2013; Lu et al. 2019). The insect proteins that interact with RBSDV P10 may be involved in viral accumulation in the insect vector. Therefore, deciphering the function of RBSDV P10 and its interaction proteins involved in the process of RBSDV transmission by L. striatellus is important to enhance the viral resistance of plants and establish a scientific system for prevention and control of RBSDV. In the present work, RBSDV P10 was used as a bait protein to screen the cDNA library of insect vector L. striatellus, and NCuP and RACK proteins interacted with RBSDV P10 directly through Y2H, LCI and pull-down assays (Figure 1). The results indicated that the interaction proteins and RBSDV P10 played vital roles in viral transmission not only in insect vectors but also in dsRNA-transgenic plants through RNAi induced by synthesized dsRNA.

Insect vectors transmit plant viruses in two ways: circulative transmission and non-circulative transmission, in which circulative viruses could cross the midgut and the epithelial barriers of accessory salivary glands in a transcytosis mechanism, where vector receptors interact with virions to complete the transmission (Gray and Gildow 2003). Evidence showed that NCuP and RACK may function in the transcytosis event of some viruses. NCuP belongs to the RR-1 family (Cornman 2009; Sinu et al. 2012). The epidermal proteins of this family mainly constitute the soft epidermis of insect tissues and have cell adhesion activity (Togawa et al. 2004). Thus, NCuP may also have cell adhesion activity. Liu (2013) found that a large number of RSV and NCuP could...
be detected in the blood cells of haemolymph of infected *L. striatellus* and RSV and NCuP have a good co-location, indicating that RSV could bind to NCuP in the blood cells of *L. striatellus*; this protein may also bind to the blood cells by interacting with RSV. Another report demonstrated that endosymbiont protein may be involved in the spread of viruses in insect body (Cilia et al. 2011). In addition, we also blasted the NCuP in NCBI to find its homologous proteins (Figure S1). When compared with other insect cuticular proteins in the NJ tree analysis with the MEGA v. 6, NCuP was not closely related to any of the other species, suggesting that it evolved independently or may be a new type of cuticular protein. RACK is another interaction protein of P10 with WD40 motif, highly homology to *Sogatella furcifera* Horváth and *Nilaparvata lugens*, and plays an important role in the regulation of various cell surface receptors and intracellular protein kinases in animals (Choi et al. 2003; Lu et al. 2019). The RACK of the vector aphid *M. persicae* could bind to WT BWYV; but not to the two mutants with low efficiency in endocytosis/transcytosis, suggesting that RACK plays a key role in this process (Seddas et al. 2004). In addition, previous studies have shown that RACK may be involved in modulating RBSDV endocytosis (Siezczarski and Whittaker 2002). Shuo et al. (2011) hypothesized that virus-binding protein RACK is potentially involved in the intercellular transport of virus. The results of the present study and those of the aforementioned studies showed that the inhibition of viral transmission and accumulation in *L. striatellus* after ingesting dsNCuP or dsRACK was likely caused by the inhibition of viral transport in insects after interfering with the interactions between both proteins and RBSDV P10. The present study further showed that RBSDV accumulation in *L. striatellus* was reduced by feeding with dsRACK. However, Lu et al. (2019) found that silencing *LsRACK1* expression in *L. striatellus* through *LsRACK1*-dsRNA microinjection enhances RBSDV accumulation. This finding may be caused by the different delivery methods of dsRNA. In addition, dsRACK mainly acts on the enteroepithelium through feeding, which affects endocytosis/transcytosis; however, dsRACK is delivered directly to the midgut barrier by injection, which activates the PKC signaling pathway (Lu et al. 2019).

The major limiting and vital factor of the RNAi process in insects is the acquisition of dsRNA. Producing dsRNA by engineering bacterial fermentation is economical; it could produce a large number of dsRNA in a short time. Tenllado and Diaz-Ruiz (2001) first discovered that the acquisition of dsRNA produced by the in-vitro expression system in non-transgenic plants could effectively interfere with viral infection. Simultaneously producing multiple dsRNAs by using a bacterial prokaryotic expression system was found to be a more effective method (Tenllado et al. 2003). Previous studies have shown that the M-JM109 strain or the M-JM109 lacY mutant strain and the vector pGEM-CP480 are the best choices for producing great quantities of dsRNA (Yin et al. 2009). In the present study, the dsRNAs of P10, NCuP and RACK were prepared using the bacterial prokaryotic expression system M-JM109 lacY/pGEM-TEasy, and the RBSDV incidence was significantly reduced after spraying rice, indicating the effectiveness of this strategy. The mechanism of a bacterial prokaryotic dsRNA expression to inhibit viral transmission mainly involves two aspects. On the one hand, the sprayed dsRNA could enter the plant cells, and it is spliced into siRNAs, which interferes with the replication and propagation of the virus in the plant. On the other hand, the dsRNA adsorbed on plant surface and siRNA generated by splicing in plant cells can also be re-fed into the body by *L. striatellus*, thus interfering with the replication and transmission of virus in insect vectors. However, this method has a short duration of protection, suggesting that multiple sprays are required to achieve good protection.

RNA-dependent RNA polymerase is an indispensable component of dsRNA amplification (Geley and Müller 2004). RdRp orthologs are present in nematodes but absent in insects (Gordon and Waterhouse 2007). Therefore, the dsRNA delivered to insects by microinjection, ingestion and soaking is due to the absence of dsRNA amplification. The plant-mediated RNAi in a transgenic plant continuously inputs high levels of dsRNA to the insect as food, thus overcoming the temporary problem of dsRNA acquisition (Wang et al. 2016; Mitter et al. 2017). Delivery of dsRNAs through transgenic plants has been studied to effectively silence genes in insects (Zhang et al. 2017). Plant-mediated RNAi has been reported in lepidopteran and coleopteran plant pests; developing this technique for application in phloem-sucking hemipteran pests, such as planthoppers, aphids and whitefly, is urgently needed because highly destructive agricultural pests cause huge yield loss and control costs worldwide (Zhongxin et al. 2019). In the present study, the target genes in *L. striatellus* could be suppressed by dsRNA ingested through diet or transgenic plants, thus inhibiting viral transmission in insects and enhancing the viral resistance of transgenic plants.

### Table 1. Resistance of transgenic rice plants expressing dsRNA to RBSDV infection.

| T2 plants* | Total number of lines | Number of lines showing different levels of resistance | Average resistance ratio (\%) |
|------------|-----------------------|------------------------------------------------------|-----------------------------|
|            | High resistance       | Middle resistance | Middle susceptibility | Susceptibility | High susceptibility | |
| P10-RNAi   | 25                    | 5                      | 5                | 9              | 6                 | 0             | 40.0%         |
| NCuP-RNAi  | 21                    | 4                      | 4                | 8              | 5                 | 0             | 38.10%        |
| RACK-RNAi  | 22                    | 3                      | 4                | 9              | 4                 | 2             | 31.82%        |
| P10+NCuP- RNAi | 24               | 8                      | 7                | 6              | 3                 | 0             | 62.50%        |
| P10+RACK- RNAi | 21             | 6                      | 5                | 6              | 4                 | 0             | 52.38%        |
| WT (L10)   | 10                    | 0                      | 0                | 0              | 0                 | 0             | 0             |

*P10-RNAi, NCuP-RNAi, RACK-RNAi, P10+NCuP-RNAi and P10+RACK-RNAi represent transgenic rice plants of T2 progeny.

**Standard:** High resistance, incidence 0.0% to 5%; Middle resistance, incidence 5.1% to 15%; Middle susceptibility, incidence 15.1% to 30%; Susceptibility, incidence 30.01% to 50%; High susceptibility, incidence >50%. The first two levels are collectively referred to as disease resistance; the last two levels are recorded as susceptibility.
The insect transmission of plant viruses needs interactions between viral proteins and vector factors (Gray and Gildow 2003; Schwarz et al. 2014). In the present work, an RNAi construct containing P10+NCuP chimeric gene, P10+RACK chimeric gene, P10 single gene, NCuP single gene, and RACK single gene was transformed into rice to investigate the viral resistance of transgenic plants. The results demonstrated that expressing the viral transmission-related gene dsRNA from the insect vector to target genes for either NCuP or RACK or the viral gene dsRNA from the virus to target P10 genes is an efficient method to develop resistance to RBSDV in transgenic rice plants. Moreover, the transgenic rice containing P10+NCuP and P10+RACK chimeric genes displayed greater resistance than the rice transformed using a single gene. Together, these results indicated that controlling viral infection in rice by simultaneously interfering with the virus-host interaction and the virus-vector interaction is practical and efficient. In addition, the success and efficiency of viral transmission in insects not only requires the specific interaction between virus and insect vectors, but also is closely related to the load and transmission of virus in insects. Therefore, in our work, the inhibition of virus transmission on transgenic plants and the emergence of disease-resistant phenotypes is likely to be associated with reduced viral load and transmissibility in insects. Considering that the transgenic plants overexpressing dsRNA of L. striatellus genes might have impact on the growth and development of L. striatellus, thus affecting the transmission of virus by vectors, leading to the reduction of the incidence of the transgenic plants. So the number of insects that survived on each particular type of plants was recorded. There was no significant difference in the number of insects on transgenic plants compared with wild-type plants. This may be due to the short duration of the insects on the transgenic plants, and the effects may not have been apparent at the time of our counted. It would be interesting to further investigate the mechanism of virus transmission by insect vectors to better explain the resistance of transgenic plants.

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Compliance with ethical standards

Human and animal rights: There was no involvement of human participants and/or animals in the present study.

Informed consent: The authors certify that this research followed the principles of ethical and professional conduct.

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