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ORIGINAL ARTICLE

Identification and characterisation of a glycine-rich RNA-binding protein as an endogenous suppressor of RNA silencing from *Nicotiana glutinosa*

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Abstract

Main conclusion **This study shows that NgRBP suppresses both local and systemic RNA silencing induced by sense- or double-stranded RNA, and the RNA binding activity is essential for its function.**

To counteract host defence, many plant viruses encode viral suppressors of RNA silencing targeting various stages of RNA silencing. There is increasing evidence that the plants also encode endogenous suppressors of RNA silencing (ESR) to regulate this pathway. In this study, using *Agrobacterium* infiltration assays, we characterized NgRBP, a glycine-rich RNA-binding protein from *Nicotiana glutinosa*, as an ESR. Our results indicated that NgRBP suppressed both local and systemic RNA silencing induced by sense- or double-stranded RNA. We also demonstrated that NgRBP could promote *Potato Virus X* (PVX) infection in *N. benthamiana. NgRBP* knockdown by virus-induced gene silencing enhanced PVX and *Cucumber mosaic virus* resistance in *N. glutinosa*. RNA immunoprecipitation and electrophoretic mobility shift assays showed that NgRBP bound to *GFP* mRNA, dsRNA rather than siRNA. These findings provide the evidence that NgRBP acts as an ESR and the RNA affinity of NgRBP plays the key role in its ESR activity. *NgRBP* responds to multiple signals such as ABA, MeJA, SA, and *Tobacco mosaic virus* infection. Therefore, it could participate in the regulation of gene expression under specific conditions.

Keywords Glycine-rich RNA-binding protein · NgRBP · RNA silencing suppressor

Abbreviations

CMV	*Cucumber mosaic virus*
EMSA	Electrophoretic mobility shift assay
ESR	Endogenous suppressor of RNA silencing
GD	Glycine-rich domain
GRP	Glycine-rich RNA-binding protein
PVX	*Potato virus X*
RBP	RNA-binding protein
RIP	RNA immunoprecipitation
RRM	RNA recognition motif
RSS	RNA-silencing suppressor
TMV	*Tobacco mosaic virus*
VSR	Viral suppressor of RNA silencing

Xu Huang and Ru Yu contributed equally to this work.

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Introduction

RNA silencing is a conserved surveillance mechanism that plays a key role in defending plants against invasive nucleic acids (Hannon 2002; Himber et al. 2003). Replication intermediates or folded viral RNAs activate RNA silencing. They are then cleaved by Dicer-like proteins (DCL) into small interfering RNAs (siRNAs) (Lichner et al. 2003; Baulcombe 2004; Susi et al. 2004). These siRNAs are incorporated into the AGO-containing RNA-induced silencing complex (RISC). Sequence-specific cleavage of target RNAs then follows (Schwarz et al. 2003; Tomari and Zamore 2005). In higher plants, RNA silencing plays a critical role in antiviral defence. To counteract RNA silencing, viruses encode RNA silencing suppressors (Baulcombe 2002). To date, RNA-silencing suppressors (RSSs) have been reported which target various effector molecules in RNA silencing (Moissiard and Voinnet 2004; Csorba et al. 2015; Daròs 2017; Yang and Li 2018). For instance, the p19 protein encoded by *Tomato bushy stunt virus* is a potent viral suppressor of RNA silencing (VSR) preventing the spread of the silencing signal by binding 21–25 nt siRNA duplexes (Vargason et al. 2003; Ye et al. 2003). In this way, it inhibits a downstream step involving the cleavage of cognate RNAs (Silhavy et al. 2002). P6, a VSR encoded by *Cauliflower mosaic virus*, interferes with viral siRNA production by interacting with double-stranded RNA-binding protein 4 (DRB4), an essential partner of DCL4 (Haas et al. 2008). Plants also encode endogenous RNA-silencing suppressors (ESRs) which function in the defence–counterdefence arm race between host plants and viruses. The first-characterized ESR, Ntrgs-CaM, was proven to interact with HC-Pro, a VSR encoded by *Tobacco etch virus,* using the yeast two-hybrid system (Anandalakshmi et al. 2000). Over the following decade, several plant ESRs were identified, including RLI2, FIERY1, XRN2, XRN3 and XRN4 from *Arabidopsis thaliana* (Gazzani et al. 2004; Sarmiento et al. 2006; Gy et al. 2007), and rgs-CaM from *Nicotiana benthamiana* (Li et al. 2014). Nevertheless, little is known about their modes of action.

Certain RSSs bind siRNA duplexes or double-stranded RNA (dsRNA) (Lakatos et al. 2006; Mérai et al. 2006). This process probably can be regulated by RNA-binding proteins (RBPs). RBPs are commonly referred to as RNA chaperones (Lorsch 2002). They have multiple members and are ubiquitous among various plant species. They directly or indirectly guide various aspects of post-transcriptional modification by interacting with specific targeted mRNAs. These interactions always require several conserved RNA-binding domains such as the RNA recognition motif (RRM), the K homology domain (KH), and the double-stranded RNA-binding domain (dsRBD) (Burd and Dreyfuss 1994). Glycine-rich RNA-binding proteins (GRPs) have two distinct conserved domains: RRM and glycine-rich domain (GD) (Gómez et al. 1988). The RRM has an octamer, ribonucleoprotein domain I (RNPI), and a hexamer, RNPII. Both of these are highly homologous. GD typically contains 2–5 glycine repeats bordered by tyrosine and/or arginine. It has been reported to be involved in protein–protein interactions (Steinert et al. 1991). The first GRP was found in maize (Gómez et al. 1988). Since then, the genes encoding homologous proteins have been consecutively isolated from a broad range of plant species such as *Medicago sativa* (Ferullo et al. 1997), *A. thaliana* (Carpenter et al. 1994) and *Sorghum bicolor* (Aneeta et al. 2002). Studies show that the circadian clock regulating RNA-binding protein AtGRP7, feedback regulates the expression of itself and its homologous gene *AtGRP8*. The binding of AtGRP7 with its own precursor mRNA (pre-mRNA) promotes its alternative splicing, and the alternative transcripts are degraded by nonsense-mediated mRNA decay (NMD). It is believed that AtGRP7 also acts on other target genes through a similar mechanism (Staiger et al. 2003; Schöning et al. 2008). In addition, AtGRP7 also participates in cold response as an RNA chaperone, and the mutant plants are more sensitive to cold. OsGRP1 and OsGRP4 can restore the growth defects of *Atgrp7* under cold stress, while OsGRP6 can enhance the frost resistance of *Atgrp7*. This indicates that the function of GRPs is somewhat conserved in dicotyledon and monocotyledon (Kim et al. 2010).

Compelling evidence indicates that GRPs mediate post-transcriptional regulation of RNA metabolism, including pre-mRNA splicing, mRNA transport, and mRNA translation. These findings present new regulatory strategies for both pathogen infection and plant defence (Fu et al. 2007; Qi et al. 2010; Jeong et al. 2011). Naqvi et al. (1998) discovered that *Tobacco mosaic virus* (TMV) induced the glycine-rich RNA-binding protein gene from *N. glutinosa NgRBP* at 24 h post-inoculation. Therefore, NgRBP may participate in plant-virus interactions. In this study, we showed that NgRBP could suppress the RNA silencing induced by sense RNA or dsRNA and prevent silencing from spreading systemically. We revealed that the RSS activity of NgRBP is associated with its RNA binding ability, and the 47th arginine residue is essential for its function.

Materials and methods

Plant materials and plasmid constructs

Wild-type *Nicotiana glutinosa*, *N. benthamiana*, and GFP-transgenic *N. benthamiana* 16c line (from Dr. David

Baulcombe, University of Cambridge, UK) (Voinnet et al. 1998) were raised in a greenhouse at 24 °C under a 16-h light/8-h dark photoperiod. The full-length open reading frame (ORF) 471-bp segment of *NgRBP* (Accession no. AF005359) was amplified from *N. glutinosa* total RNA by RT-PCR using DNA Polymerase High Fidelity (HiFi) (TransGen, Beijing, China). The PCR product was ligated to a pMD-18T vector to generate pMD-NgRBP. All NgRBP mutants including ΔRNPI, ΔRNPII and R47A were produced by reverse PCR from the entire plasmid pMD-NgRBP, using the primer pairs that contained the corresponding nucleotide substitution or deletion (Table S1). The constructs were cloned into the binary vector pBI121 between the 35S promoter and the Nos terminator (Chen et al. 2003). For transient expression in 16c plants, 35S-p19, 35S-GFP, and 35S-dsGFP were constructed as previously described (Jing et al. 2011). To generate *NgRBP* carrying *Potato Virus X* (PVX) vector, *NgRBP* was inserted into the *Cla* I/*Not* I site in the PVX vector (pGR106) (Voinnet et al. 2000). All fragments generated by PCR were confirmed by DNA sequencing. The recombined plasmids were transformed by the freeze–thaw method into *Agrobacterium* strain GV3101 which contains the helper plasmid pJIC SA Rep (Höfgen and Willmitzer 1988).

Co-infiltration and GFP imaging

The 16c plants expressing GFP were infiltrated at five- or six-leaf stage with *Agrobacterium* GV3101 carrying the aforementioned constructs using the previously described method (Brigneti et al. 1998). Each *Agrobacterium* culture ($OD_{600}=1.0$) was incubated for 3 h and then mixed with other culture (s) in a 1:1 (v/v) ratio prior to infiltration. Local and systemic RNA silencing were determined by observing GFP fluorescence both in the infiltrated and newly emerging leaves under long-wavelength (365 nm) UV light (Spectroline Model SB-100P/A; Spectronics Corporation, Lexington, KY, USA) and photographed with a Fujifilm FinePix S8000fd digital camera (Fujifilm Holdings Corporation). More than five plants were tested per experimental condition.

Virus-induced gene silencing (VIGS)

For the *NgRBP* VIGS, TRV-based vectors were used as previously described (Chung et al. 2004). To ensure specific silencing of *NgRBP*, the full-length CDS was inserted into the TRV2 vector to generate the pTRV2:*NgRBP*, and there is no homologous gene in *N. glutinosa* by NCBI Blast. The pTRV1, pTRV2:00 (empty vector), and pTRV2:*NgRBP* constructs were transformed into *Agrobacterium* GV3101. The *Agrobacterium* cultures were resuspended in infiltration buffer containing 10 mM $MgCl_2$, 10 mM Mes, and 200 mM acetosyringone (pH 5.6; $OD_{600}=0.8$). *Agrobacterium* culture harbouring pTRV1 was mixed with pTRV2:00 or pTRV2:*NgRBP* in a 1:1 ratio and then infiltrated into the lower leaves of 3-week-old *N. glutinosa* plants. The infiltrated plants were then placed in an illuminated incubator at 24 °C and 70% RH under a long-day (16-h light/8-h dark) photoperiod. After 15 days post-inoculation (dpi), the empty vector control plants and the *NgRBP*-silenced plants were used in Real-time quantitative polymerase chain reaction (RT-qPCR) and PVX or CMV inoculation assays.

For virus inoculation, 1 g of PVX (PVX-HM isolate, GenBank GQ863228) or CMV (CMV-SD1 isolate, GenBank AY792596)-infected *N. tabacum* leaves were ground in 1 mL of 5 mM phosphate buffer, pH 7.2. Control plants and the *NgRBP*-silenced plants at six-leaf stage were inoculated by rubbing leaves with freshly prepared sap. Inoculated plants were grown in an insect-free greenhouse at 24 °C and the viral symptom was monitored. Each experiment was replicated three times and each experiment included five independent plants.

RT-qPCR analysis

Total RNAs were isolated from leaves using Trizol reagent (TaKaRa, Shiga, Japan) according to the manufacturer's instructions and treated with DNase I at 37 °C for 30 min prior to reverse transcription. cDNA was synthesized from 1 µg of total RNA using TIANScript RT Kit (Tiangen, Beijing, China). RT-qPCR was performed using Talent SYBR Green Kit (Tiangen, Beijing, China). The *ssRUBP* gene was used as the internal reference. Each reaction was conducted in triplicate and repeated three times. The results were analysed by Bio-Rad CFX Manager software (Bio-Rad, California, USA).

Total RNA and siRNA Northern blot analysis

Total and low molecular weight RNAs were extracted from leaves as described previously (Jing et al. 2011). 20 µg total RNA aliquot of each sample was separated on 1% formaldehyde agarose gels and transferred to Hybond-N^+ membranes (GE Healthcare, Marborough, USA) by upward capillary transfer in 20 × SSC buffer. The membranes were hybridized with digoxigenin (DIG)-labelled probes corresponding to the full-length ORFs of *GFP*, *NgRBP*, *PVX-CP* and *CMV-2b*, respectively. For siRNA detection, 15 µg low molecular weight RNAs were separated on 15% polyacrylamide–7 M urea gel and transferred to a Hybond-N^+ membrane in 0.5 × TBE at 0.8 mA cm^{-2} for 1 h. After being UV-crosslinked and incubated at 80 °C for 2 h, the membrane was hybridized with DIG-labelled probe *GFP* mRNA. Chemiluminescent detection was conducted using a DIG

Northern Starter Kit (Roche, Basel, Switzerland) according to the manufacturer′s instructions.

Electrophoretic mobility shift assay (EMSA)

His-tagged NgRBP, R47A, NS1 and p19 proteins were expressed in *Escherichia coli* strain BL21 and purified by His-Tagged Protein Purification Kit (CwBio, Beijing, China). The *GFP* mRNA and dsRNA were created by T7 RiboMAXTM Express RNAi System (Promega, Wosconsin, USA) using specific primers *GFP* sense RNA F/R and *GFP* anti-sense RNA F/R in Table S1. Two complementary 21 nt *GFP* siRNAs with 2-nt 3′ overhangs (*GFP* siRNA-F, CUG UCCACACAAUCUGCCCUU; *GFP* siRNA-R, GGGCAG AUUGUGUGGACAGUU) were synthesized by Takara. *GFP* siRNAs were annealed to obtain double-stranded siRNAs. EMSA assay was performed using a Light Shift RNA EMSA Optimization and Control Kit (Thermo, Massachusetts, USA) according to the manufacturer's instructions. The binding reactions were incubated for 30 min at room temperature. The products were separated on an 8% polyacrylamide gel and transferred to a nylon membrane. The membrane was then hybridized with DIG-labelled GFP mRNA probes. Chemiluminescent detection was conducted using a DIG Northern Starter Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

RNA immunoprecipitation assay (RIP)

Three grams of leaves co-infiltrated with *Agrobacterium* carrying 35S-GFP with 35S-His-NgRBP, 35S-His-R47A or empty vector were put in 50-mL conical centrifuge tubes containing 37 mL of 1% formaldehyde and 2.5 mL of 2 M glycine separately, and vacuumed for 15 min in a closed container attached to a vacuum pump. Then the samples were ground and 30 mL extraction buffer 1 was added (0.4 M sucrose, 10 mM Tris–HCl, 5 mM BME, 0.1 mM PMSF). Solutions were filtered through two layers of 200 mesh stainless steel filter screen, and centrifuged at 1252*g* for 20 min at 4 °C. Pellets were resuspended in 1 mL extraction buffer 2 (0.25 M sucrose, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM BME, 0.1 mM PMSF), and centrifuged at 9469*g* for 10 min at 4 °C. Then pellets were resuspended in 600 µL extraction buffer 3 (1.7 M sucrose, 10 mM Tris–HCl, 0.15% Triton X-100, 2 mM $MgCl_2$, 5 mM BME, 0.1 mM PMSF), spined at the top speed in for 1 h at 4 °C, and resuspended in 500 µL nuclear lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 1% SDS, 0.1 mM PMSF). Sample solutions were ultrasonicated on ice for 30 min. 60 µL supernatants are added to 540 µL RIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, 167 mM NaCl) and 25 µL equilibrated protein A beads and mixed on a shaker at 4 °C for 1 h. Then 5 µL His antibody (TransGen, Beijing, China) was added to each tube, and incubated on a shaker rotating mixer for 4 h at 4 °C. Immune complexes were eluted with 500 µL elution buffer (100 mM $NaHCO_3$, 1% SDS) for 30 min at 65 °C. Crosslinking was reversed at 65 °C for 1 h with 20 µg proteinase K (Invitrogen, California, USA). RNA was purified by acidic phenol, chloroform and ethanol precipitation. RT-qPCR was used to detect the enriched levels of each segment of *GFP* mRNAs using the primers in Table S1.

Results

Expression profiles of NgRBP gene in *N. glutinosa*

NgRBP gene encodes a GRP harbouring a single copy of RRM which comprising highly conserved RNPI (R-G-F-G-F-V-T-F) and RNPII (C-F-V-G-G-L) at the N terminus and a GD at the C terminus. Sequence alignment indicated that a high level of amino acid similarity spanning the whole protein sequence exists among NgRBP homologs, suggesting that these proteins are evolutionarily conserved (Fig. 1a). Several GRPs were also isolated in other plant species, including *A. thaliana*, *N. tabacum* and *Solanum lycopersicum*. Phylogenetic analysis indicated that NgRBP shared a high amino acid similarity (94.3%) to NtGRP1 (Accession no. ACD03270) (Fig. 1b), a negative regulator of gene expression through binding to DNA or RNA under flooding stress (Lee et al. 2009). A well-studied glycine-rich RNA-binding protein AtGRP7 (Accession no. Q03250) shows 76% similarity to NgRBP (Fig. 1b), having an RRM in N terminal and a GD in C-terminal.

The tissue-specific expression of *NgRBP gene* was analysed by Northern blot. As shown in Fig. 2a, *NgRBP* gene is constitutively expressed in all examined organs, and clearly expressed at higher levels in root and stem compared with leaf and flower. To further study the expression regulation of *NgRBP* gene, a 982-bp fragment of its 5′-flanking region was isolated from *N. glutinosa* genomic DNA using inverse PCR technique. Three types of *cis*-acting element were predicted to be related to environment response: ABA-responsive element, methyl jasmonate (MeJA)-responsive element and light-responsive element. In addition, the *cis*-acting element required for endosperm expression was also predicted within the promoter region of *NgRBP* (Fig. S1). Expression profiles by Northern blot analysis proved that the *NgRBP* expression was indeed induced by ABA and MeJA in *N. glutinosa* leaves. The *NgRBP* transcripts were detectable at 4 h, reached peak level at 8 h and gradually decreased by 12 h after ABA treatment (Fig. 2b), while the *NgRBP* expression gradually increased from 4 to 24 h after MeJA

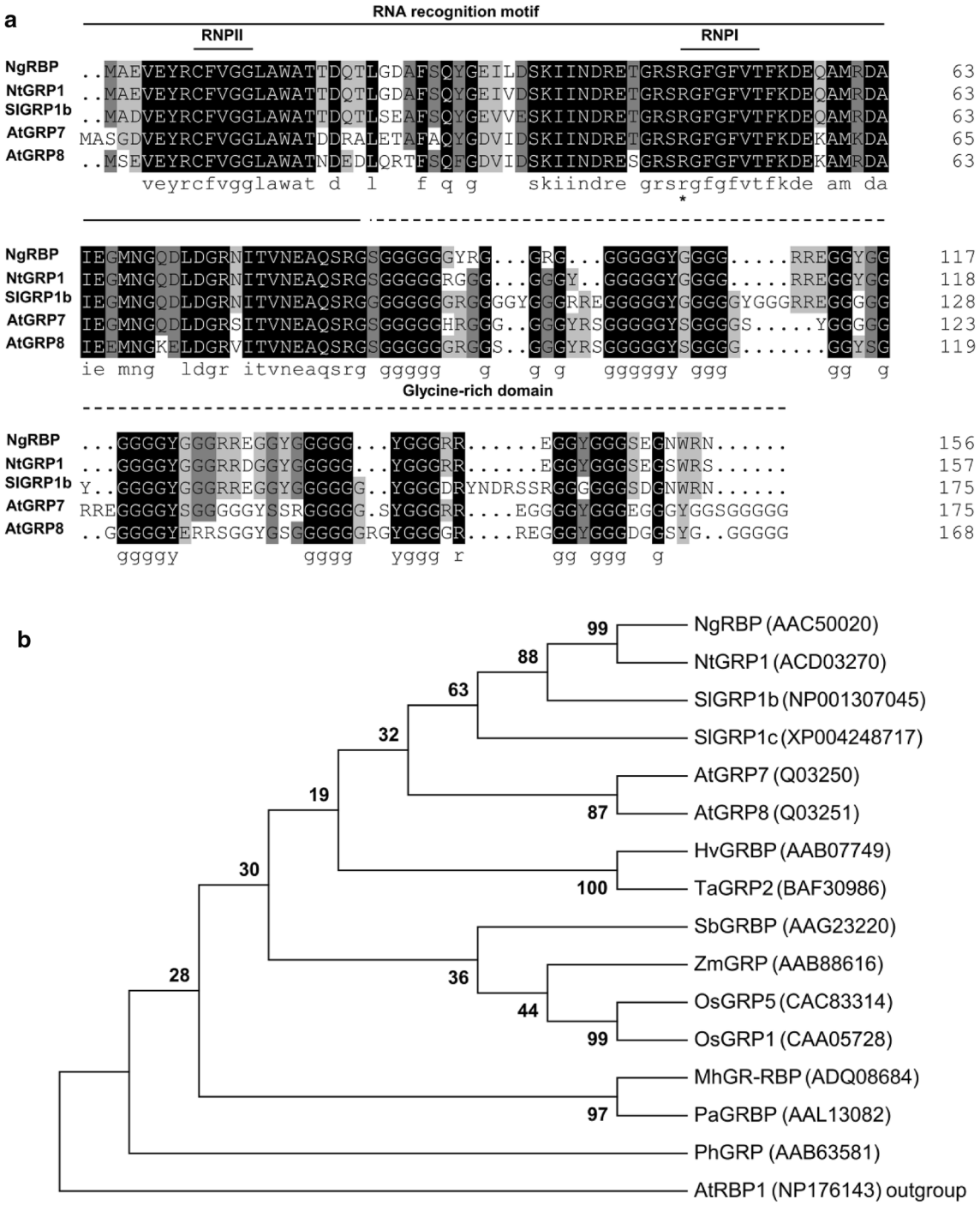

Fig. 1 Structure and conservation of NgRBP. **a** Pairwise alignment of GRBPs from *N. glutinosa* (NgRBP, AF005359), *N. tabacum* (NtGRP1, EU569289), *S. lycopersicum* (SlGRP1b, JQ613216), *A. thaliana* (AtGRP7, Q03250 and AtGRP8, Q03251) using DNAStar. Identical and similar regions were indicated by a black background. The N-terminal RRM harbouring two highly conserved regions, RNPI and RNPII, was denoted by a solid line above its sequences. The C-terminal GD was represented by dotted lines. The 47th amino acid was marked with an asterisk. **b** Phylogenetic analysis showed the relationship of NgRBP and its homologues from various plant species. The tree was generated in MEGA with the neighbour-joining algorithm (1000 replicates) using nucleotide sequences (see supporting information). Bootstrap values were shown near the internal nodes. AtRBP1, a glycine-rich RNA-binding protein from *Arabidopsis* unrelated to NgRBP in sequence, was used as an out group

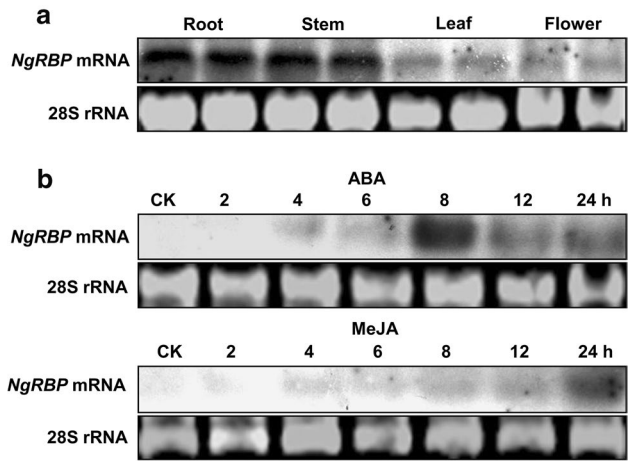

Fig. 2 *NgRBP* expression profiles in *N. glutinosa*. **a** Tissue-specific *NgRBP* expression was detected by Northern blot analysis using total RNAs extracted from the roots, stems, leaves, and flowers of *N. glutinosa* plants. Ethidium bromide-stained rRNA was used as a loading control. **b** Northern blot analysis of the *NgRBP* expression induced by ABA and MeJA. Total RNAs were extracted from leaves at the indicated time after treatment with 100 μM ABA and 1 mM MeJA, respectively. Control plants were sprayed with water. Ethidium bromide-stained rRNA was used as a loading control

treatment (Fig. 2b). In addition, Naqvi et al. (1998) showed that *NgRBP* was also induced by salicylic acid (SA; Naqvi et al. 1998). It suggests NgRBP might be involved in the responses to biotic and abiotic stresses.

NgRBP blocks both local and systemic RNA silencing triggered by GFP sense RNA or dsRNA

The effect of *NgRBP* gene on RNA silencing was investigated using *Agrobacterium*-mediated transient expression system in GFP-transgenic *N. benthamiana* line 16c plants. *Agrobacterium* strain harbouring *NgRBP* was mixed with the *Agrobacterium* carrying *GFP*, and infiltrated into the leaves of 16c plants. The co-infiltrations of GFP with an empty vector or with p19 were performed as negative and positive controls. GFP fluorescence was hardly observed in the infiltrated leaves with empty vector at 6 dpi under UV light, but the patches infiltrated with NgRBP displayed high-intensity GFP fluorescence at that time, similar to the infiltration of p19 (Fig. 3a). Northern blot analysis revealed that, both at 3 dpi and 6 dpi, the *GFP* mRNA levels of the leaves infiltrated with NgRBP plus GFP or p19 plus GFP were obviously higher than the leaves infiltrated with GFP plus empty vector. The siRNA blots also confirmed that NgRBP drastically reduced the *GFP*-specific siRNAs both at 3 dpi and 6 dpi, similar to p19 (Fig. 3b).

Furthermore, the effect of NgRBP on systemic spread of RNA silencing was studied. 16c plants infiltrated with above *Agrobacterium* combinations were further observed to monitor GFP expression in the newly emerged leaves at 12 dpi. As previously reported, systemic GFP silencing was visualized as the disappearance of the GFP fluorescence in the GFP-infiltrated plants (Voinnet et al. 1998; Silhavy et al. 2002).

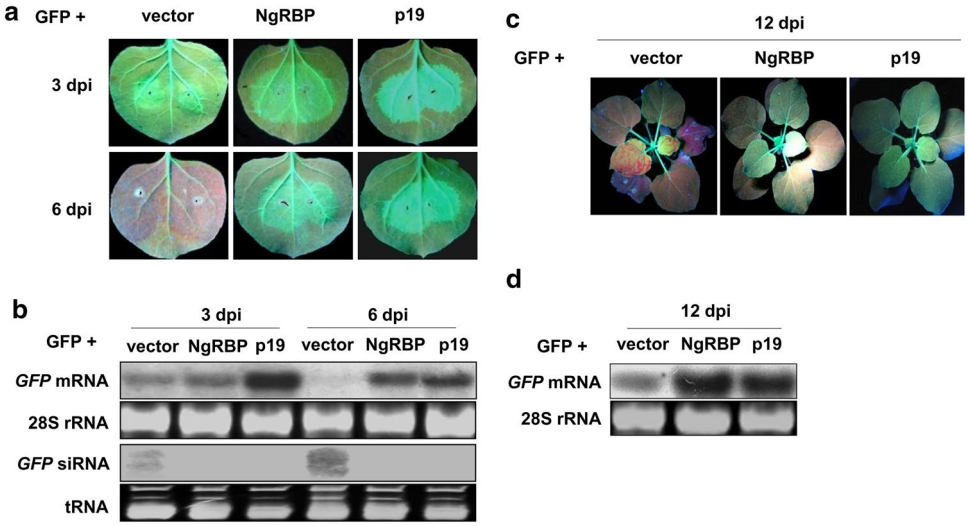

Fig. 3 NgRBP blocks both local and systemic RNA silencing triggered by sense GFP RNA. **a** Suppression of local GFP silencing in GFP-transgenic *N. benthamiana* line 16c. Leaf patches were co-infiltrated with *Agrobacterium* cultures expressing GFP (35S-GFP) and a vector control, NgRBP or TBSV p19. Photographs of 16c leaves were taken at 3 dpi and 6 dpi under handheld long-wave ultraviolet lamp. **b** Northern blot analysis of *GFP* mRNA and siRNA extracted at 3 dpi and 6 dpi from patches co-infiltrated with the various strains indicated above each lane. Ethidium bromide-stained rRNA and tRNA were used as loading controls for mRNA and siRNA, respectively. **c** Photographs of 16c plants infiltrated with *Agrobacterium* harbouring 35S-GFP and a vector control, NgRBP or TBSV p19 under handheld long-wave ultraviolet lamp at 12 dpi. **d** Northern-blot analysis of *GFP* mRNA isolated from systemic leaves of plants with the different strains indicated above each lane at 12 dpi. Ethidium bromide-stained rRNA was used as a loading control

Most of the newly emerged leaves of plants infiltrated with NgRBP plus GFP still maintained GFP fluorescence, similar to that of p19 (Fig. 3c). The levels of steady-state *GFP* mRNA in the newly emerged leaves were consistent with the GFP fluorescence observed under UV light (Fig. 3d). Taken together, these results demonstrate that NgRBP has a bona fide ESR activity and inhibits RNA silencing induced by sense GFP RNA both at local and systemic levels.

RNA silencing is a multi-step process (Lichner et al. 2003; Baulcombe 2004; Susi et al. 2004). To determine in which step NgRBP targets RNA silencing, we tested the effect of NgRBP on *GFP* dsRNA-triggered silencing. Leaves of 16c plants were co-infiltrated with the *Agrobacterium* carrying NgRBP, GFP plus dsGFP. We found that the patches co-infiltrated with GFP plus dsGFP showed red fluorescence under UV light at 6 dpi. But GFP fluorescence still sustained when NgRBP was added (Fig. 4a). High *GFP* mRNA levels and negligible *GFP*-specific siRNA were detected by Northern blot analysis in the infiltrated leaves with NgRBP (or p19) addition (Fig. 4b). To further demonstrate whether NgRBP interferes with systemic RNA silencing triggered by dsGFP, we co-infiltrated 16c leaves with GFP and dsGFP plus NgRBP. Our results showed that, at 12 dpi, systemic RNA silencing occurred in the dsGFP infiltrated plants with trace amounts of *GFP* mRNA accumulated. But GFP fluorescence was maintained in the newly emerged leaves of the infiltrated plants with NgRBP or p19 addition (Fig. 4c), which was consistent with the levels of *GFP* mRNA detected by Northern blot analysis (Fig. 4d). This indicated that NgRBP also suppressed local and systemic RNA silencing triggered by dsRNA.

NgRBP increases the pathogenicity of PVX and *NgRBP* silencing confers enhanced resistance to PVX and CMV

To investigate whether NgRBP increases viral pathogenicity, the PVX vector pGR106 was utilized to express NgRBP in *N. benthamiana* plants (Fig. 5a). At 10 dpi, plants infected with PVX-*NgRBP* showed more severe disease symptoms along the veins than those receiving PVX vector alone (Fig. 5b). Viral RNAs accumulated to a higher level in PVX-NgRBP-inoculated plants than those infected with PVX (Fig. 5c).

We investigated the role of *NgRBP* in plant defence responses using the *Tobacco rattle virus* (TRV)-based VIGS technique. *NgRBP* silencing resulted in milder symptoms than that of the controls (Fig. 6a). The RT-qPCR showed that the *NgRBP* mRNA level in TRV:*NgRBP* plants was ~ 70% lower than that of the empty vector controls (TRV:00) at 15 dpi (Fig. 6b), indicating that *NgRBP* was effectively silenced in *N. glutinosa* plants. Systematically *NgRBP*-silenced plants were then inoculated with PVX and CMV, respectively. Symptoms were visibly different between the

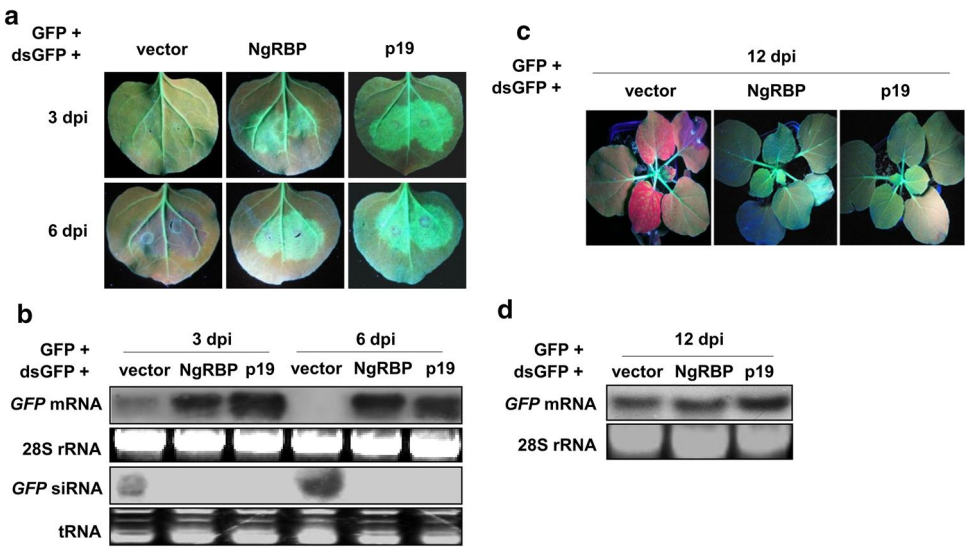

Fig. 4 NgRBP suppresses local and systemic RNA silencing triggered by dsGFP RNA. **a** 16c leaves were co-infiltrated with three strains of *Agrobacterium* carrying 35S-GFP, 35S-dsGFP, and either empty vector, NgRBP or TBSV p19. The GFP expression in the infiltrated tissue was monitored at 3 dpi and 6 dpi under handheld long-wave ultraviolet lamp. **b** Northern-blot analysis of *GFP* mRNA and siRNA extracted from the patches with the different strains indicated above each lane at 3 dpi and 6 dpi. Ethidium bromide-stained rRNA and tRNA were used as the loading controls for mRNA and siRNA, respectively. **c** Photographs of 16c leaves infiltrated with *Agrobacterium* harbouring 35S-GFP, 35S-dsGFP and either vector control NgRBP or TBSV p19 under handheld long-wave ultraviolet lamp at 12 dpi. **d** Northern blot analysis of *GFP* mRNA isolated from systemic leaves of the plants with the different strains indicated above each lane at 12 dpi. Ethidium bromide-stained rRNA was used as a loading control

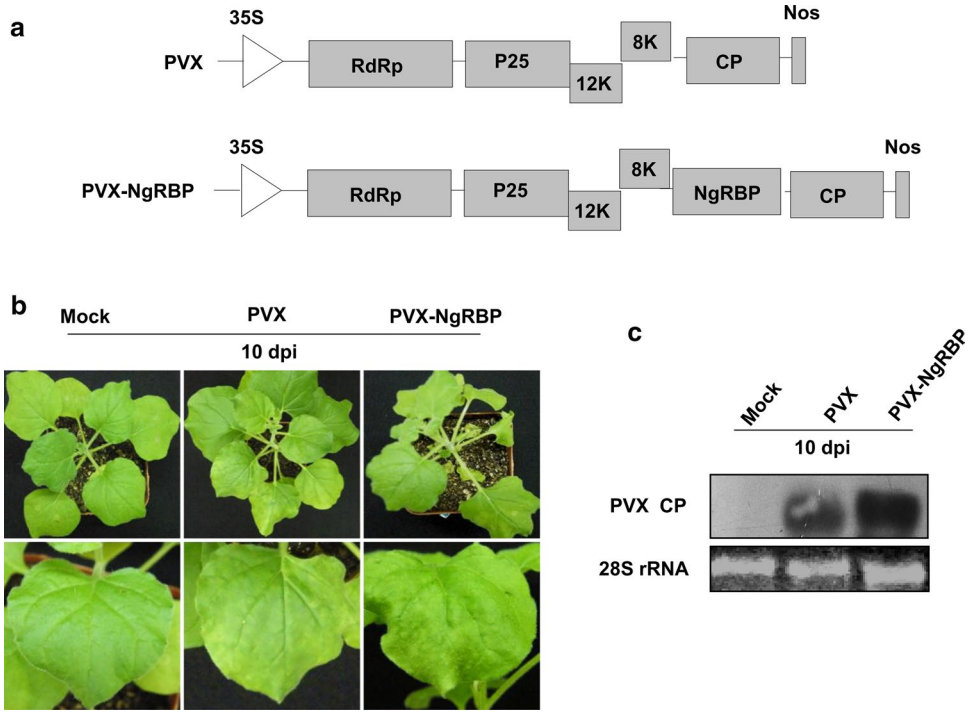

Fig. 5 NgRBP enhances the pathogenicity of chimeric PVX. **a** Schematic representation of recombinant PVX variants carrying NgRBP. **b** Symptoms in *N. benthamiana* plants infected with the recombinant PVX variants. More than 5 plants that were tested. Mock plants were infiltrated with buffer only. Systemic leaves (bottom panels) were photographed at 10 dpi. **c** Northern blot analysis of the accumulation of PVX genomic RNA in the systemic leaves at 10 dpi. The bottom panels show rRNA with ethidium bromide staining as a loading control

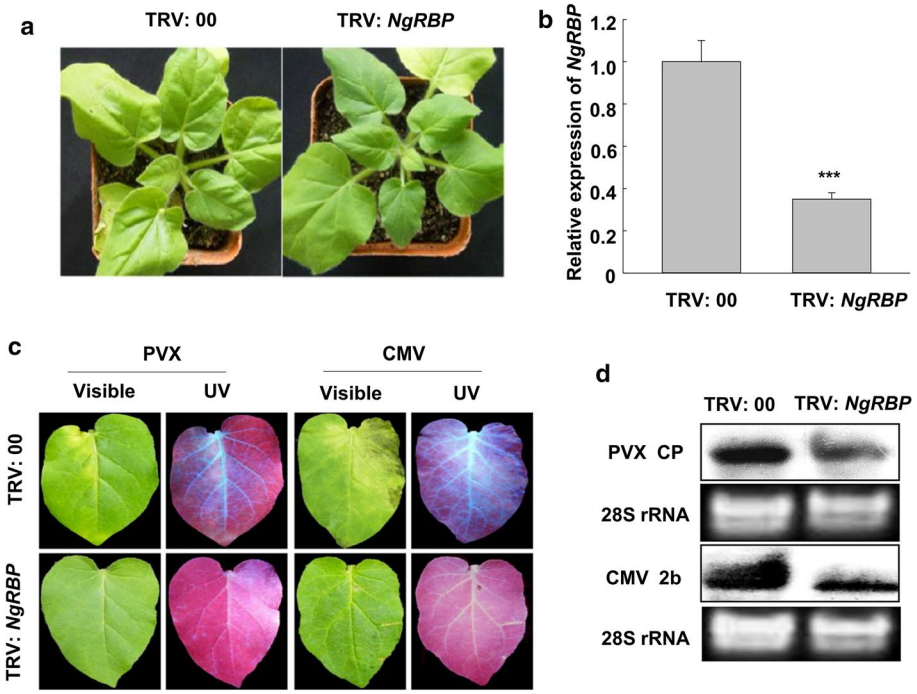

Fig. 6 *NgRBP* silencing in *N. glutinosa* plants with enhanced resistance to PVX and CMV. **a** Phenotype of *NgRBP*-silenced and empty vector control plants. Photographs were taken at 10 dpi. **b** RT-qPCR analysis of *NgRBP e*xpression in the empty vector control (TRV: 00) and the *NgRBP*-silenced (TRV:*NgRBP*) plants. **c** Visible- and ultraviolet lamp-illuminated disease symptoms developed on the empty vector control and the *NgRBP*-silenced plants infected with PVX (left panel) and CMV (right panel). Photographs were taken at 10 dpi. **d** Northern blot analysis of the accumulation of PVX genome RNA and CMV RNA2 at 10 dpi. Ethidium bromide-stained rRNAs were used as loading controls

NgRBP-silenced plants and the controls. The latter were relatively more susceptible to PVX and CMV infection than the former. Only slight mosaicism and downward leaf curling were observed in the *NgRBP*-silenced plants. UV illumination revealed that the control plants infected with PVX and CMV accumulated more phenolic compounds than the *NgRBP*-silenced plants did (Fig. 6c). Northern blot analysis showed that the levels of PVX genomic RNA and CMV RNA2 were higher in the control plants than in the *NgRBP*-silenced plants at 10 dpi (Fig. 6d). These results indicate that NgRBP interferes with antiviral RNA silencing in host plants.

The RNA binding activity of NgRBP is essential for its RNA silencing suppressor function

Much evidences support the idea that the silencing suppression activity of RSSs commonly requires the ability of RNA binding (Lakatos et al. 2006; Mérai et al. 2006). Two conserved RNA-binding motifs in RRM domain of GRPs, RNPI and RNPII, particularly the R47 residue of RNPI, play the key role in RNA recognition and binding (Khan et al. 2014). Therefore, two deletion mutants ΔRNPI and ΔRNPII, and a point mutant R47A in which the 47th arginine being substituted by alanine were constructed to explore the functional domain and loci of NgRBP using *Agrobacterium* transient expression system. Strong GFP fluorescence was observed in the tissues co-infiltrated with 35S-GFP and ΔRNPII constructs at 3 dpi, similar to that of NgRBP co-infiltration. The ΔRNPI and R47A constructs yielded weak fluorescence at 3 dpi (Fig. 7a). Then, to explore whether the RSS function of NgRBP is related to its RNA binding activity, NgRBP and R47A proteins were purified (Fig. S2) and incubated with *GFP* mRNA. EMSA assays showed that NgRBP rather than R47A bound to *GFP* mRNA (Fig. 7b). To study dsRNA binding ability of NgRBP, NS1 was used as a positive control in the EMSA assay, which is an *Avian influenza virus*-encoded RSS with siRNA and longer dsRNA affinity (Lin et al. 2007; Yu et al. 2018). The result showed that NgRBP could bind to *GFP* dsRNA, but R47A could not. Furthermore, RIP assays showed that NgRBP protein could enrich about fivefold 3′ end fragments of *GFP* mRNA compared with the control and the R47A protein (Fig. 7c). siRNA affinity of NgRBP was also detected by EMSA with p19 as a positive control (Silhavy et al. 2002). The result showed that NgRBP does not bind to siRNA which usually was taken as target by many VSRs (Fig. 7d). These results suggested that RNA binding activity of NgRBP is essential for its RSS function, and the 47th arginine located in RNPI motif is the key functional site.

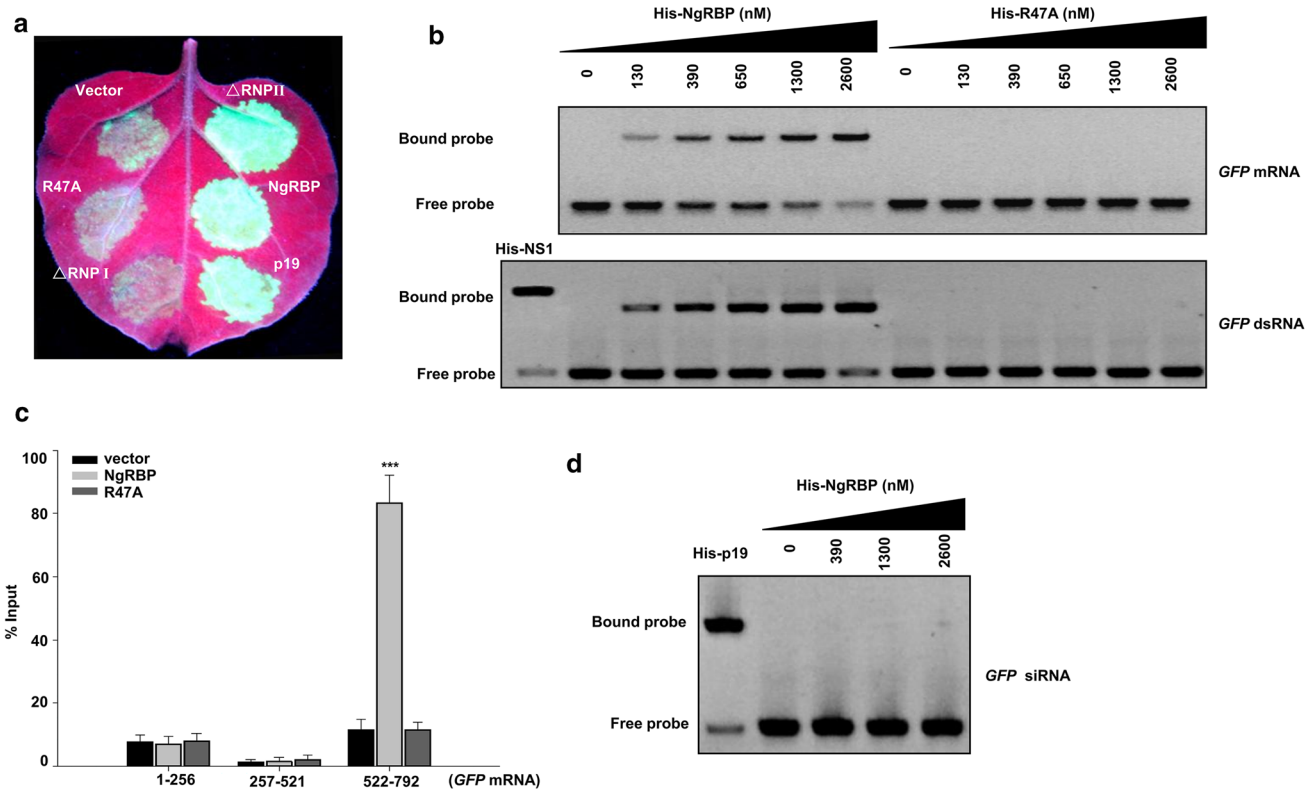

Fig. 7 NgRBP binds to the 3′ end of *GFP* mRNA and dsRNA but not siRNA. **a** Co-infiltration in *N. benthamiana* leaves with GFP and empty vector, NgRBP, ΔRNPI, ΔRNPII, R47A or p19, respectively. Images were obtained under handheld long-wave ultraviolet lamp at 3 dpi. **b** EMSA analysis of samples contained 1 μM *GFP* mRNA or *GFP* dsRNA and in addition 0, 130, 390, 650, 1300, and 2600 μM His-NgRBP and His- R47A protein, respectively. 390 μM NS1 was used as a positive control. **c** RIP analysis of *GFP* mRNA extracted from patches co-infiltrated GFP with His-NgRBP, His-R47A or empty vector at 3 dpi. **d** EMSA assay of samples contained 1 μM *GFP* siRNA and in addition 0, 390, 1300, and 2600 μM His-NgRBP, respectively. The free probe bands correspond to the siRNA duplexes. 390 μM p19 was used as a positive control

Discussion

A study on *N. tabacum* rgs-CaM provided the earliest clue that host genes served as negative regulators of RNA silencing (Anandalakshmi et al. 2000). Later, several ESRs or endogenous negative factors involved in RNA silencing have been discovered by genetic screening. It is believed that ESRs always induce the expression of certain silenced genes depending on developmental requirements. They may also be subverted by viruses in counterattacks against host antiviral responses (Trinks et al. 2005; Sarmiento et al. 2006; Gy et al. 2007; Endres et al. 2010). Recent studies showed that rgs-CaM had opposite effect on RNA silencing-mediated antiviral defence compared with its ESR function. So rgs-CaM was proposed as a counter-VSR factor that quenched certain VSRs containing dsRBD (Nakahara et al. 2012). HC-Pro was proved to upregulate the antiviral silencing potentially antagonists, such as *Arabidopsis* FRY1 and CML38 (a homologue of rgs-CaM) which both being defined as ESRs. rgs-CaM, FRY1, and CML38 are thought to be the downstream mediators of HC-Pro (Anandalakshmi et al. 2000; Gy et al. 2007; Endres et al. 2010). These findings suggested that the endogenous silencing pathway was subject to negative feedback regulation in certain cases. VSRs may require an ESR either to activate their components or to be able to work together to block silencing.

In this study, we characterized NgRBP, an RNA-binding protein from *N. glutinosa*, as a novel ESR. NgRBP could suppress local and systemic RNA silencing induced by sense RNA or dsRNA. NgRBP belongs to the class IVa of GRPs with the typical structure including an RRM in N terminal and a GD in C-terminal. Our data showed that when RNPI, one of the conserved motifs of RRM in NgRBP, was deleted, NgRBP lost its RSS activity. R47 mutation in RNPI motif brought the same effect on its function, and the mutant protein no longer bound to *GFP* mRNA. It is suggested that RNA binding ability of NgRBP effectively contributes to its RSS activity. A recent report revealed that in *Arabidopsis* both 5′-3′ and 3′-5′ cytoplasmic RNA decay pathways act as repressors of transgene and endogenous PTGS to safeguard plant transcriptome and development (Zhang et al. 2015; Zhang and Guo 2017). AtXRN4 which being in charge of 5′-3′ mRNA degradation antagonizes RNA silencing possibly by degrading the template for RdRp or interacting with HC-Pro (Gazzani et al. 2004; Li and Wang 2018). Our results showed that NgRBP could bind to the 3′ end of *GFP* mRNA and dsRNA, it is speculated that NgRBP might prevent RdRp from dsRNA synthesis at the early stage of RNA silencing and, therefore, impede the formation of RISC complex, or inhibit the step of Dicer processing by competitive dsRNA binding, respectively. Similar strategies are adopted by some viral RNA silencing repressors, such as *Infectious bursal disease virus* (IBDV) VP3 (Valli et al. 2012), *Turnip crinkle virus* (TCV) p38 (Iki et al. 2017), and *Flock house virus* (FHV) B2 (Lingel et al. 2005).

Why would a host plant have evolved proteins to thwart its own defence mechanisms? The ESR regulatory mechanism is required to prevent exaggerated RNA silencing, ensuring that appropriate PTGS in the plants. ESRs may potentially impair the host silencing machinery controlling cellular RNA levels so that they are normally suppressed until required. This postulate is supported by the observation that viral infection or wounding induces certain ESRs (Naqvi et al. 1998; Tadamura et al. 2012). Plants GRPs have been identified in a variety of plant species, and have been demonstrated to be regulated by a number of external stimuli including hormones and pathogens (Kang et al. 2013). As a TMV-induced GRP, *NgRBP* was induced by ABA, MeJA, SA as well (Naqvi et al. 1998; Czolpinska and Rurek 2018). Therefore, the suppressor function of NgRBP is most likely closely associated with the defence or multiple stress responses. Previous researches showed that virus attack induced GRPs expression in tobacco, petunia and rice, and some of them contributed to virus resistance (van Kan et al. 1988; Linthorst et al. 1990; Fang et al. 1991; Ueki and Citovsky 2002). AtGRP7, a typical GRP with 76% similarity to NgRBP, plays a positive role in the defence against TMV (Lee et al. 2012) and is described to preferentially bind to 3′UTR of target genes (Staiger et al. 2003; Meyer et al. 2017). It is similar to our findings that NgRBP could bind to the 3′ end of *GFP* mRNA. Both AtGRP7 and AtGRP8, the NgRBP homologs, were proved to be involved in an interlocked feedback loop through which they could autoregulate and reciprocally cross-regulate by coupling unproductive splicing to nonsense-mediated mRNA decay (NMD; Schöning et al. 2008). NMD is a host RNA control pathway that removes aberrant transcripts due to premature termination of codons that always arises through defective splicing. RNA viruses often contain internally located stop codons that should also be prime targets for NMD (May et al. 2018). We observed that NgRBP transient overexpression in *N. glutinosa* enhanced TMV resistance. Further studies need to explore the exact mechanisms of NgRBP involved in viral defence and stress response.

Author contribution statement HL designed the research and edited the paper; XH and RY performed the main experiments and drafted the paper; WL, LG, XJ and CZ revised the paper. All authors have read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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