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RNA silencing is activated by *N* gene-mediated hypersensitive response and plays a key role in local and systemic virus resistance

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Abstract

In plants, recognition between resistance gene (*R*) and virus induces a local hypersensitive response (HR), which is accompanied by systemic acquired resistance (SAR). The dominant resistance gene *N* in tobacco confers resistance to tobacco mosaic virus (TMV) at both locally inoculated tissues and systemically infected tissues. However, the mechanisms underlying HR- and SAR-mediated viral inhibition are not fully revealed. In this study, we find that *Nicotiana glutinosa* RNA-binding protein (NgRBP) is an RNA silencing suppressor which enhances TMV-triggered HR. Stronger HR could result in stronger local and systemic RNA silencing as well as SAR. Enhanced RNA silencing in the systemically infected leaves induced by the *NgRBP* gene is compromised by transient expression of *NahG*. These results indicate that RNA silencing is activated by HR and plays a crucial role in local and systemic virus resistance. Our results reveal a crosstalk between *N* gene-mediated virus resistance and RNA silencing which would deepen our understanding of the established HR and SAR models.

Keywords TMV, *N* gene, RNA silencing, Hypersensitive response, Systemic acquired resistance

Background

The hypersensitive response (HR) and systemic acquired resistance (SAR) are essential components of plant defence mechanisms against pathogens. HR against virus infection is always initiated upon recognition of virus avirulence factors by plant resistance (R) proteins. Most dominant *R* genes recognizing viruses condition broad-scale changes in plant biochemistry and physiology that are activated and regulated by various signal transduction pathways. These induced changes include localized cell death and the upregulation of resistance against many types of pathogens throughout the plant. This is conveyed via a signal transduction cascade involving MAP kinases, reactive oxygen species (ROS) and nitric oxide (NO) production, leading to programmed cell death (Jones & Dangl 2006, Mur et al. 2006, Fu & Dong 2013).

After HR, SAR is initiated by a signal transmitted from the local infection site to distal tissues, providing systemic protection against secondary infections (Mur et al. 2006). This concerns the generation of mobile

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signals like salicylic acid (SA), methyl salicylate (MeSA), and lipid-transfer proteins (Vlot et al. 2009, Klessig et al. 2018). SAR leads to the transcriptional reprogramming of a wide array of defence-related genes. This involves not only pathogenesis-related protein (PR) genes but also genes involved in fortifying cell walls, detoxifying ROS, and other stress-related responses (Fu & Dong 2013). Studies indicate that SAR also involves epigenetic modifications, including histone modifications and DNA methylation, which play a part in the regulation of defence gene expression (Luna & Ton 2012). SA accumulates in the tissues where SAR is activated and binds to nonexpressor of pathogenesis-related proteins 1 (NPR1) to activate the expression of *PR* genes throughout the plant (Durrant & Dong 2004, Choudhary et al. 2007). Despite considerable research on the mechanisms of HR and SAR, it is still poorly understood how virus infection is inhibited and restricted during the HR in the initially infected tissues and in the systemically infected tissues exhibiting SAR (Carr et al. 2010).

The *N* gene confers resistance to TMV and encodes a TIR-NBS-LRR protein (Whitham et al. 1994). The N protein is proved to recognize the 50 kD helicase domain of TMV replicase mediated by the protein NRIP1 (Burch-Smith et al. 2007). This recognition triggers a signal transduction cascade that induces HR and limits virus transmission (Abbink et al. 1998, Zhang et al. 2019). The local HR often leads to SAR characterized by non-specific general defence response throughout the plant (Ryals et al. 1996). *N* gene-mediated recognition activates downstream signal transduction pathways involving various plant hormones, such as SA and jasmonic acid (JA), and triggers programmed cell death, thereby limiting the spread of the pathogen in local tissues. This is a strategy of sacrificing local cells to protect the whole plant (Jones & Dangl 2006). Our previous study showed that NgRBP, an RNA-binding protein encoded by *Nicotiana glutinosa* (NN), could enhance TMV resistance (Huang et al. 2019). In this study, HR, SAR, and RNA silencing in TMV-infected *N. glutinosa* were investigated using *NgRBP*. The results showed that the TMV-induced HR-activated RNA silencing which could spread to the systemically infected leaves of *N. glutinosa*. This indicates that RNA silencing is involved in virus-induced HR and SAR. As a transmissible and broad-spectrum antiviral mechanism, RNA silencing might play a key role in virus restriction and inhibition in SAR.

Results

NgRBP promotes HR and SAR induced by TMV

Local TMV infection induces SAR in plants harbouring the *N* gene, which confers resistance to TMV via gene-for-gene interactions. We observed that transient expression of *NgRBP*, an endogenous RNA silencing suppressor encoded by *N. glutinasa* (Huang et al. 2019), enhanced HR induced by TMV infection. To investigate whether the local resistance enhanced by *NgRBP* affects the SAR, *NgRBP* and an empty vector were transiently infiltrated into *N. glutinasa* leaves separately. One day later, the infiltrated leaves of the plants were inoculated with TMV, and their upper leaves were re-inoculated with TMV at 3 d post-inoculation (dpi) (Fig. 1a). The results showed that the necrotic spots in the systemically infected leaves of *NgRBP*-infiltrated plants were smaller than those in the (empty vector) control plants (Fig. 1c). Using the virus-induced gene silencing (VIGS) technology, the effect of *NgRBP* on virus resistance was further investigated. *N. glutinosa* leaves were first infiltrated with tobacco rattle virus (TRV)-*NgRBP*, and TMV was inoculated twice in the upper leaves at 7 and 11 dpi; TRV vector infiltration was used as a control (Fig. 1b). The necrotic spots on the upper leaves of *NgRBP* VIGS plants were larger and more intense than those in the controls (Fig. 1c). RT-qPCR showed that TMV accumulation levels in the systemically infected leaves were highest in *NgRBP* VIGS plants, followed by the empty vector control, and lowest in *NgRBP* infiltrated plants (Fig. 1d). Gene expression analysis showed that the expression levels of *PR* genes in the three treatments were inversely related to the TMV accumulation levels (Fig. 1e). These results indicated that *NgRBP* enhanced the SAR induced by TMV infection.

To investigate whether the function of HR enhancement is specific to *NgRBP*, two other viral RNA silencing suppressors (RSSs), P19 and HVT063, whose RSS activity has been reported previously (Silhavy et al. 2002, Jing et al. 2011), were applied to detect their effects on TMV-induced HR. *P19, HVT063*, and *NgRBP* were transiently expressed in *N. glutinosa* leaves, with empty vector infiltration as a control. One day later, TMV was mechanically inoculated into the infiltrated regions. Necrotic symptoms were observed at 3 dpi of TMV, and the leaves were sampled for virus accumulation detection using RT-qPCR for the TMV capsid protein (CP) gene. Similar to *NgRBP*, *P19* and *HVT063* expression resulted in fewer and smaller necrotic lesions compared with the empty vector controls (Fig. 2a). RT-qPCR showed less TMV accumulation in *P19* and *HVT063* expressing leaves than in the controls (Fig. 2b). All three detected RSSs enhanced HR, suggesting that RSS activity may be responsible for increased resistance, as inhibiting RNA silencing in the primary stages of TMV infection ensures that there is enough virus level to trigger stronger HR. This finding aligns with the results obtained by Pruss et al. (2004), who showed that TMV infection in HC-Pro-expressing plants carrying the *N* gene leads to a reduced

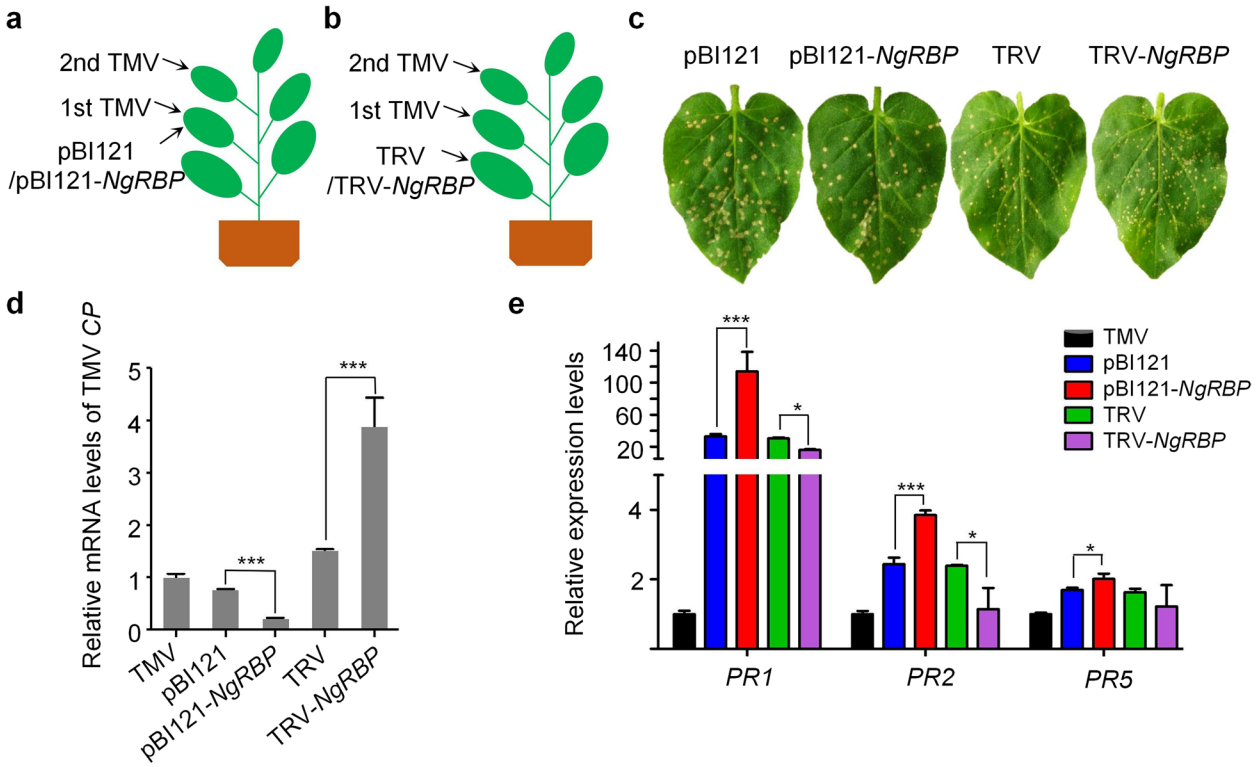

Fig. 1 *NgRBP* enhances systemic acquired resistance (SAR) triggered by tobacco mosaic virus (TMV) infection in *Nicotiana glutinosa*. **a** Schematic representation of two times TMV inoculation in *NgRBP* transient-infiltrated plants. pBI121-NgRBP and empty vector were transiently infiltrated in *N. glutinasa* leaves separately; thereafter, TMV was inoculated in the infiltrated leaves at 1 d post-infiltration (dpi). At 3 dpi, the infiltrated plants were reinoculated with TMV in their upper leaves (the third leaf from the top), and the symptoms were observed 3 days later. **b** Schematic representation of two TMV inoculations in *NgRBP* VIGS plants. Firstly, TRV-NgRBP and empty TRV vectors were infiltrated in *N. glutinosa* leaves, respectively. TMV was inoculated in the upper leaves at 7 dpi, and TMV was reinoculated in the new upper leaves after 4 days. **c** Symptom observation of the second TMV challenge. **d** Virus accumulation analysis using TMV capsid protein (CP) of qRT-PCR. **e** Levels detection of *PR1*, *PR2*, and *PR5* using qRT-PCR. Gene expression values of the plants that were directly inoculated with TMV were set at 1.0. The mRNA levels of the analyzed genes were normalized by *Actin1*, which served as an internal standard. The data were analyzed using Student's *t*-test, and asterisks denote significant differences compared with control plants (* $P \leq 0.05$, *** $P \leq 0.001$)

number and size of lesions compared with the control plants.

Gene expression profiling of enhanced SAR mediated by *NgRBP*

To determine the effect of *NgRBP* on SAR, gene expression profiling was performed using RNA-seq. *N. glutinosa* leaves were infiltrated with *NgRBP* and inoculated with TMV one day later. Empty vector-infiltrated and TMV-inoculated plants were used as controls. RNA sequencing profiles were obtained from the upper leaves of these plants at 3 dpi of TMV. Using a false discovery rate threshold of 0.05 and an absolute Log_2 fold change ≥ 1 ($P \leq 0.05$) as the significance cutoff, 1002 differentially expressed genes (DEGs) were found to be significantly regulated by *NgRBP* compared with controls. Overall, 833 genes were upregulated and 169 downregulated (Additional file 1: Figure S1).

To match the functional enrichment of commonly up- or down-regulated genes during SAR enhancement, gene ontology (GO) enrichment analysis was performed. Common overrepresented GO terms are shown in Additional file 1: Figure S2, and 19 significant GO terms were identified. A total of 1002 DEGs were divided into cellular component (CC), molecular function (MF), and biological process (BP) categories according to their GO functional annotations. The proportion of DEGs in each subclass of the above categories was counted. Among these, the BP category was significantly enriched in cellular protein metabolic processes (GO:0044267) (156) and macromolecular modifications (GO:0043412) (126). Among the MF GO terms, phosphotransferase activity, alcohol group as an acceptor (GO:0016773) (89), kinase activity (GO:0016301) (88), and protein kinase activity (GO:0004672) (83) were considerably enriched. No subclass was enriched in CC categories (Additional file 1: Figure S2). Similar to the GO functional annotation, 1002

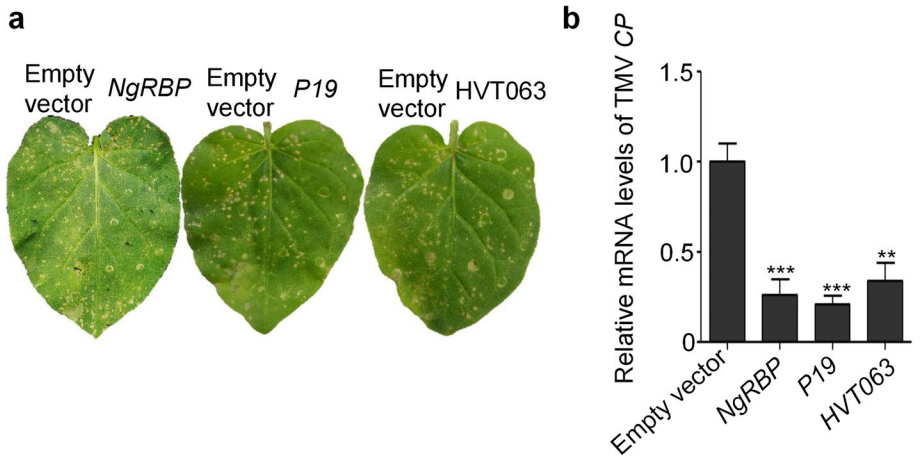

Fig. 2 *P19* and *HVT063* enhance systemic acquired resistance triggered by tobacco mosaic virus (TMV) infection in *Nicotiana glutinosa*. **a** Observation of necrotic symptoms. **b** Virus accumulation analysis. First, pBI121-*NgRBP,* pBI121-*P19,* and pBI121-*HVT063* (right) or empty vectors (left) were infiltrated into *N. glutinosa* leaves, and one day later, TMV was mechanically inoculated in the above-infiltrated leaves. At 3 d post-infiltration of TMV inoculation, necrosis symptoms in the infiltrated area were observed, and the tissues of the infiltrated area were sampled for virus accumulation analysis using TMV capsid protein (CP) of RT-qPCR. The mRNA levels of *CP* were normalized to those of *Actin1,* which served as an internal standard. The data were analyzed using Student's t-test, and asterisks denote significant differences compared with control plants (** $P \leq 0.01$, *** $P \leq 0.005$)

DEGs from *NgRBP* and control groups were annotated using the Kyoto Encyclopedia of Genes and Genomes database. Sixty-nine enriched pathways were identified, of which five were significantly increased ($P < 0.05$). The most enriched was the ribosomal pathway (KO00940), which included 20 DEGs. The second step involved protein processing in the endoplasmic reticulum containing 11 DEGs. Furthermore, ten DEGs were involved in plant and pathogen interactions, eight in phenylpropanoid biosynthesis, and six in ribosome biogenesis in eukaryotes (Additional file 2: Table S1).

Eight resistance-related genes that were upregulated in *NgRBP*-expressing plants, based on RNA-seq data, were selected for RT-qPCR analysis (Additional file 2: Table S2). These genes showed higher levels in the systemically infected leaves of *NgRBP*-expressing plants than in the controls. Among these, *PRR1*, *RGA3*, and *PRRP2* were significantly upregulated (Fig. 3a). To further study the effect of *NgRBP* on SAR, nine SAR-related genes were selected. The proteins encoded by these genes were divided into six categories: leucine-rich repeat protein (LRR), cell wall-related proteins including glycine-rich protein precursor, reactive oxygen species-related proteins including glutathione S-transferase, pathogen-related proteins including PR-1a, SAR induced protein 8.2e (SAR8.2) family which responds to agents that induce SAR, and SAR marker genes including *PR1*, *PR2*, *PR5*, and *NPR1*. Previous studies indicated that LRR1 as a receptor-like protein kinase might play a role in signal transduction in the resistance response of tobacco to TMV (Jacques et al. 2006). The results showed that the expression of these genes was upregulated in *NgRBP*-expressing plants compared with that in the controls. Among them, *PR1*, *PR2*, *PR5*, *LRR1*, and *SAR8.2e* levels were significantly increased in the *NgRBP* group (Fig. 3b). These results indicated that *NgRBP* promotes the expression of resistance-related genes in systemically infected leaves where SAR occurs and plays a key role in SAR enhancement.

TMV-induced HR enhances both local and systemic RNA silencing

RNA silencing is a natural plant defence mechanism against viruses. It is triggered by double-stranded RNAs (dsRNAs), formed during viral replication, and inhibits viral amplification by specifically degrading viral RNAs. To determine the effect of TMV infection on RNA silencing, *N. glutinosa* leaves were transiently infiltrated with *Agrobacterium* carrying the *GFP* gene, and TMV was inoculated into the infiltrated leaves the next day. Simultaneously, the upper leaves were infiltrated with *Agrobacterium* carrying *GFP* gene. GFP fluorescence of the lower and upper leaves was observed under UV light at 6, 12, 24, 36, 48, and 60 h post-TMV inoculation (hpi), respectively. Compared with the control (the leaves mock-inoculated with PBS buffer), the GFP fluorescence of TMV-inoculated leaves was weaker at all observation times, especially at 36 and 48 hpi. At 36 hpi, fluorescence induced by cell necrosis appeared in TMV-inoculated leaves. At 60 hpi, TMV infection resulted in large cell

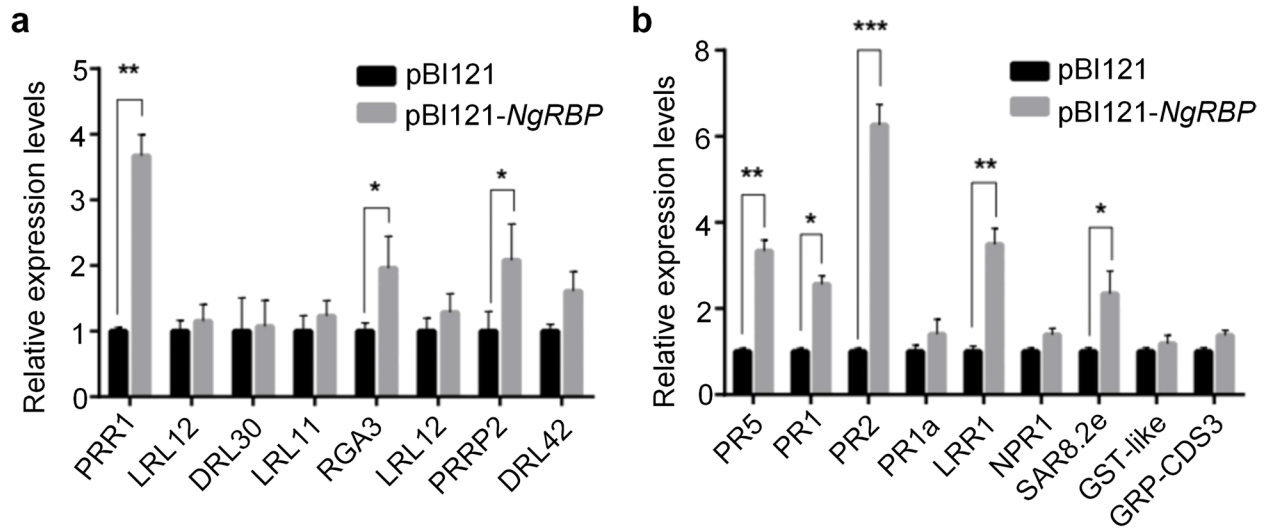

Fig. 3 The expression levels of disease resistance-related genes in systemic *Nicotiana glutinosa* leaves. **a** Expression detection of the up-regulated disease resistance-related genes in RNA-seq using RT-qPCR. **b** Expression detection of systemic acquired resistance (SAR)-related genes. pBI121-NgRBP and pBI121 were infiltrated in *N. glutinosa* leaves, respectively, and tobacco mosaic virus was infiltrated one day later. The upper uninfected leaves were sampled to extract total RNA at 3 d post-infiltration. The expression levels of disease resistance-related genes selected from differentially expressed genes and SAR-related genes were detected by RT-qPCR. The standard deviation was based on the results of three biological repetitions. The data were analyzed using Student's *t*-test, and asterisks denote significant differences between the two groups (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$)

necrosis areas (Fig. 4a). The GFP fluorescence of the upper leaves of TMV-inoculated plants was weaker at all observation times compared with that of the mock-inoculated plants (Fig. 4b). These results suggest that TMV-induced HR enhances both local and systemic RNA silencing in *N. glutinosa*.

Enhanced HR is associated with stronger local RNA silencing

Our previous study showed that *NgRBP* is an endogenous silencing suppressor (Huang et al. 2019), and the transient expression of *NgRBP* in *N. glutinosa* leaves could promote HR induced by TMV infection. To explore the role of *NgRBP* in TMV defence, *GFP* and *NgRBP* or empty vector were co-infiltrated into *N. glutinosa* leaves, and TMV was inoculated into the co-infiltrated leaves. Changes in the GFP fluorescence over time were observed under UV light. At 6 hpi, stronger fluorescence was observed in the co-infection area of *GFP* and *NgRBP* compared with *GFP* and empty vector co-infiltration, although both fluorescence intensities were relatively weak. This difference may be due to the silencing-suppressive activity of *NgRBP*. From 12 to 48 hpi, GFP fluorescence in the area co-infiltrated with *GFP* and *NgRBP* became weaker than that of the empty vector control. Necrosis was observed at 48 hpi in TMV-inoculated area, and GFP fluorescence in both treatments disappeared at 60 hpi (Fig. 5a).

Co-infiltrated tissues were sampled at different time points for Northern blot analysis to verify this observation. The results showed that *GFP* mRNA levels were consistent with the fluorescence observations. *GFP* siRNA analysis showed little accumulation in *GFP* and empty vector co-infiltrated tissues at any time. In contrast, small amounts of siRNA were detected in *GFP* and *NgRBP* co-infiltrated leaves at 24, 36, and 48 hpi, and *GFP* siRNA levels increased with time (Fig. 5b). At 60 hpi, no accumulation of *GFP* siRNA was detected in any of the co-infiltrations, which may have been caused by cell death induced by the HR response. These results suggest that the stronger the HR, the stronger the local RNA silencing.

HR mediated by *N* gene prompts systemic RNA silencing

To investigate the effect of HR on systemic RNA silencing, pBI121-*NgRBP* and an empty vector were transiently infiltrated into *N. glutinosa* leaves. One day later, infiltrated leaves were inoculated with TMV and infiltrated with *Agrobacterium* carrying *pBI121*-GFP (Fig. 6a). In addition, *N. glutinosa* was infiltrated with *Agrobacterium* carrying TRV-*NgRBP* and empty vectors. After 7 d, the systemic leaves were inoculated with TMV, and the upper leaves were infiltrated with pBI121-*GFP* simultaneously (Fig. 6b). GFP expression in each treatment was monitored under UV light 3 d after pBI121-*GFP* infiltration. It was observed that the GFP

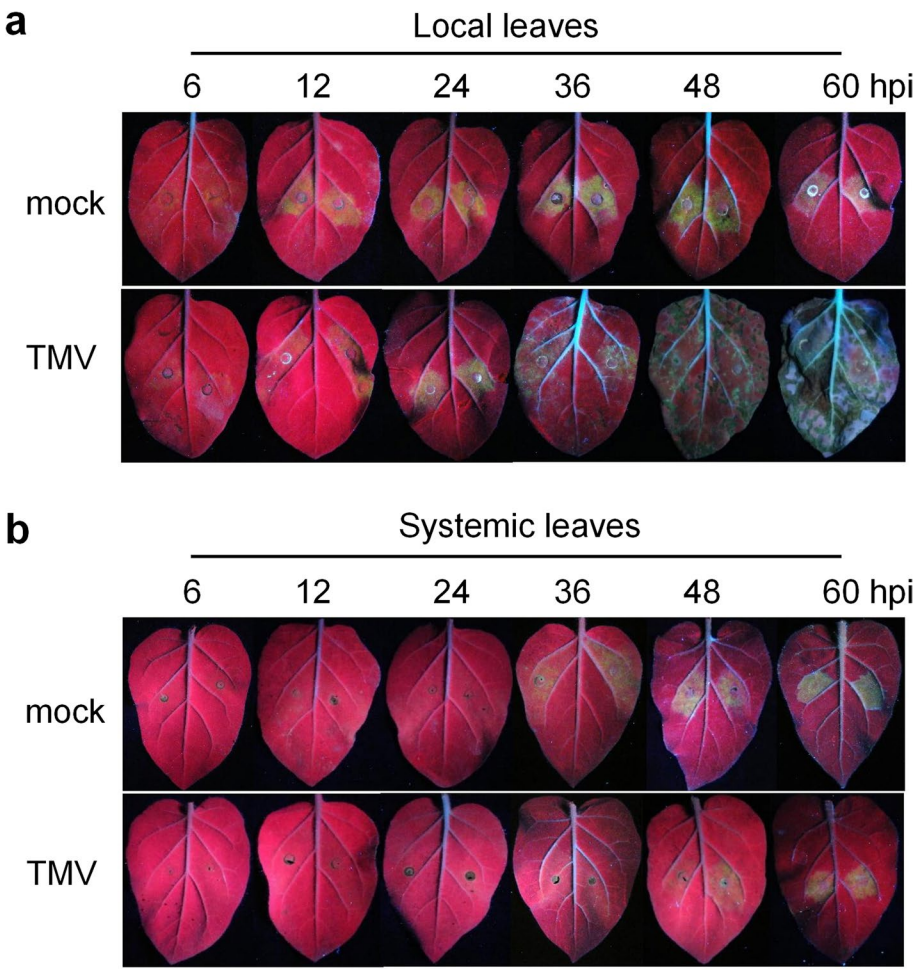

Fig. 4 The effect of tobacco mosaic virus (TMV) infection on RNA silencing in GFP transient-expressing leaves of *Nicotiana glutinosa*. **a** GFP fluorescence of GFP transiently infiltrated and TMV inoculated leaves. First, the leaves were infiltrated with *Agrobacterium* carrying GFP, and one day later, TMV was inoculated into the GFP-infiltrated leaves. GFP fluorescence was observed under a long-wave UV lamp at 6, 12, 24, 36, 48, and 60 h post-TMV inoculation (hpi), respectively. **b** GFP fluorescence in the systemic leaves of *N. glutinosa* plants whose lower leaves were inoculated with TMV or PBS. The lower leaves of the plants were inoculated with TMV, and simultaneously, the upper leaves were infiltrated with *Agrobacterium* carrying GFP. GFP fluorescence was observed under a long-wave UV lamp at 6, 12, 24, 36, 48, and 60 hpi, respectively

fluorescence of pBI121-*NgRBP* infiltration was weaker than that of the empty vector control, and the GFP fluorescence of TRV-*NgRBP* infiltration was more potent than that of TRV alone (Fig. 6c). Northern blot analysis at 3 dpi showed that the mock-inoculation and TMV inoculation treatments accumulated a large amount of *GFP* mRNA in the infiltrated zone; whereas the other treatments showed little *GFP* mRNA accumulation in the infiltrated zones. *GFP* mRNA accumulation in pBI121-*NgRBP* was slightly lower than that of empty pBI121, and *GFP* mRNA of TRV-*NgRBP* was slightly higher than that of TRV. A large amount of *GFP* siRNA was detected in the systemically infected leaves of the pBI121-*NgRBP* and empty vector TRV-infiltrated plants. A small amount of *GFP* siRNA accumulated in the systemically infected leaves of pBI121 and TRV-*NgRBP* infiltrated groups. *GFP* siRNAs in TMV-infected and mock-inoculated plants without *Agrobacterium* infiltration were almost undetectable (Fig. 6d). These results suggest that *NgRBP*-enhanced HR also prompts systemic RNA silencing in *N. glutinosa*.

To verify the effect on RNA silencing, the systemic leaves of *N. glutinosa* plants with *NgRBP* expression and TMV infection were sampled for RT-qPCR. The results showed that the detected genes in the RNA silencing pathway were upregulated compared to those in the empty vector control. Among them, *DCL4* and *AGO7* were significantly upregulated (Fig. 7a). Previous studies have reported that SA plays a crucial role as an SAR signal to activate resistance to various pathogens,

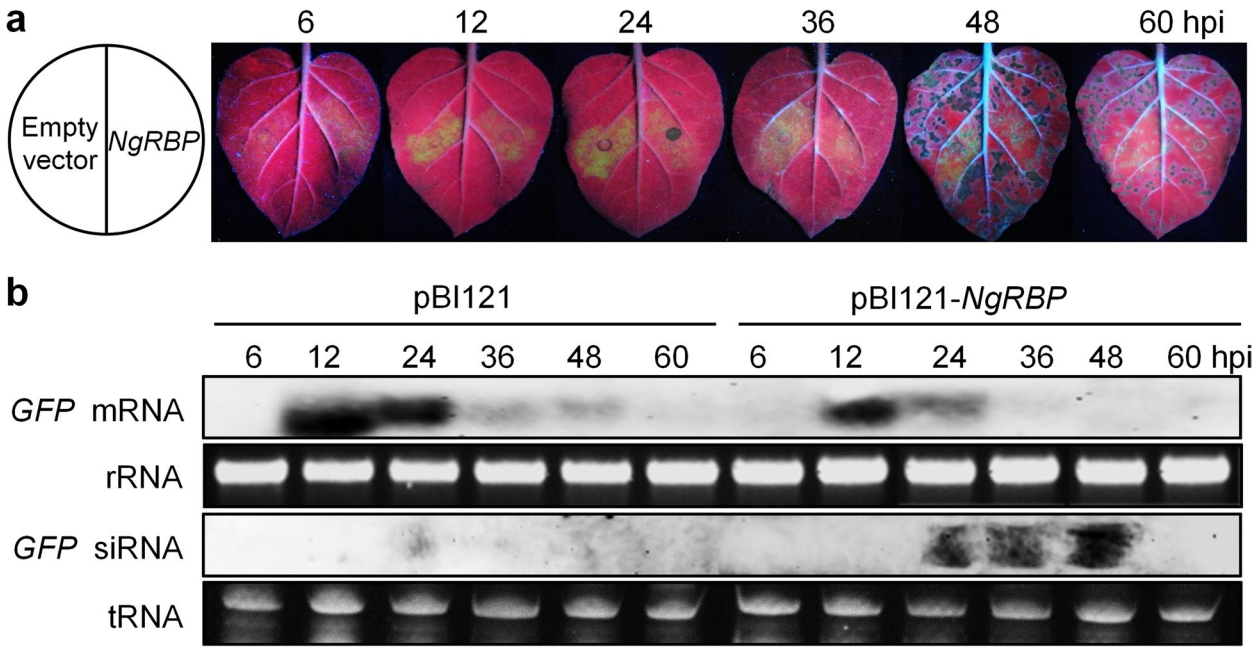

Fig. 5 *NgRBP* enhances local RNA silencing triggered by tobacco mosaic virus (TMV) infection. **a** GFP fluorescence observation under UV illumination. **b** Detection of *GFP* mRNA and siRNA accumulation using Northern blot. First, pBI121-GFP together with pBI121-NgRBP or an empty vector were co-infiltrated into *Nicotiana glutinosa* leaves, and 1 d later, TMV was inoculated in the above co-infiltrated leaves. The change of GFP fluorescence with time was observed under UV illumination at 6, 12, 24, 36, 48, and 60 h post-TMV inoculation. Total RNA and small RNA from the co-infiltrated patches were extracted for Northern blot analysis. Ethidium bromide-stained rRNAs and tRNAs were used as loading controls for mRNA and siRNA analyses, respectively

including viruses (Thomma et al. 1998, Métraux et al. 1990). To determine whether SA affects RNA silencing mediated by HR, *NgRBP* and TMV were infiltrated or inoculated into *N. glutinosa* leaves, and thereafter *NahG*, a bacterial gene encoding salicylate hydroxylase that can significantly decrease SA accumulation by converting it to catechol (Yamamoto et al. 1965) together with *GFP*, was transiently expressed in systemically infected leaves. The results showed that GFP fluorescence was weaker in the upper leaves of the plants where *NgRBP* was locally expressed before TMV infection than in the empty vector control. However, when *NahG* was transiently expressed in systemically infected leaves, the weaker GFP fluorescence was restored (Fig. 7b). These results indicate that SA may play a key role in systemic RNA silencing activated by local *NgRBP* transient expression together with TMV inoculation.

Discussion

R gene-virus interactions always result in local acquired resistance (LAR) and SAR. Although there have been many studies on cytopathy and signal transduction in HR and SAR, the mechanisms of LAR and SAR remain unclear. The localized infection mediated by HR is a highly effective mechanism that enables plants to resist infection by viruses. The zone surrounding a TMV lesion exhibits resistance to not only TMV but also other viruses (Kang et al. 2005). Numerous cytopathic changes lead to the collapse and necrosis observed during TMV-induced HR. This process begins with a reactive oxygen burst (Allan et al. 2001), followed by swelling and distortion of the chlorophyll lamella (Weintraub & Ragetli 1964), increased electrolyte leakage, and a notable increase in the superoxidic radical monohydroascorbate (Fodor et al. 2001). HR involves various physiological and biochemical processes, including programmed cell death (PCD), reversible-protein phosphorylation, and protein degradation (Dunigan & Madlener 1995, Lacomme & Santa Cruz, 1999, del Pozo & Lam 2003). Furthermore, during HR, there is a sharp increase in the levels of several hormones such as SA, methyl jasmonate (MeJA), and ethylene (Mur et al. 1997, Malamy et al. 1990, Darby et al. 2000). None of these changes occur in systemically infected susceptible plants.

Some hypotheses have been raised to explain virus localization in HR, which believe that the death of cells in a necrotic lesion may localize or inactivate the virus. However, this explanation is not satisfactory, as virus particles are found in apparently viable cells outside the necrotic areas (Murphy et al. 2001, Wright et al. 2000).

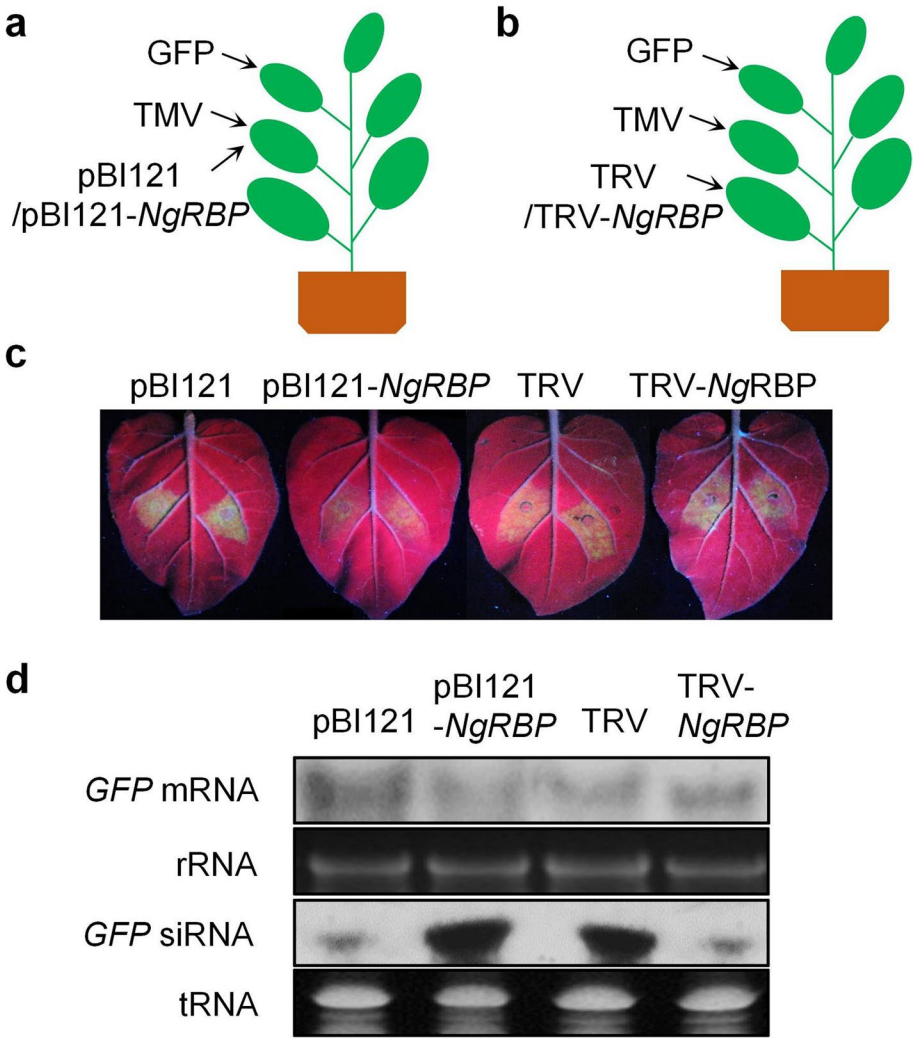

Fig. 6 *NgRBP* enhances systemic RNA silencing triggered by tobacco mosaic virus (TMV) infection. **a** Schematic representation of TMV inoculation together with transiently up-regulated *NgRBP*. **b** Schematic representation of TMV inoculation together with VIGS down-regulated *NgRBP*. **c** GFP fluorescence observation under UV illumination of *NgRBP* up-regulated and down-regulated leaves. **d** Detection of *GFP* mRNA and siRNA accumulation using Northern blot of *NgRBP*-up-regulated and down-regulated *N. glutinosa* leaves. pBI121-*NgRBP* and the empty vector were transiently infiltrated to *N. glutinosa* leaves, respectively. One day later, the above-infiltrated leaves were inoculated with TMV, and simultaneously, the upper leaves were infiltrated with *Agrobacterium* carrying pBI121-*GFP*. First, *Agrobacterium* carrying TRV-*NgRBP* and empty vectors were infiltrated into *N. glutinosa* leaves. The upper leaves were inoculated with TMV at 7 d post-infiltration (dpi), and the upper leaves were infiltrated with pBI121-*GFP* simultaneously. At 3 dpi, GFP fluorescence was observed under UV illumination, and total RNA and small RNA of the co-infiltrated patches were extracted for Northern blot analysis. Ethidium bromide-stained rRNA and tRNA were used as loading controls for mRNA and siRNA analyses, respectively

More research support the viewpoint that HR-induced cell death and inhibition of virus replication are two different processes (Loebenstein et al. 1980, Loebenstein & Gera 1981, Cole et al. 2001). Barrier substances such as callose were also suggested to be involved in virus localization (Wu & Dimitman 1970). However, callose depositions were also observed in systemic necrosis, where the virus does not remain localized (Shimomura & Dijkstra 1975). Canto and Palukaitis (2002) found that cucumber mosaic virus (CMV) 1a gene could prompt TMV spread in *NN* tobacco. While CMV 1a protein maintains the silencing suppressor activity of the 2b protein and might affect virus restriction in HR through regulating RNA silencing (Watt et al. 2020). Based on our findings, we suggest that the TMV-induced HR activates RNA silencing, which plays a crucial role in LAR. RNA silencing has been established as a robust mechanism that can directly degrade the genomic RNA of plant viruses. Therefore,

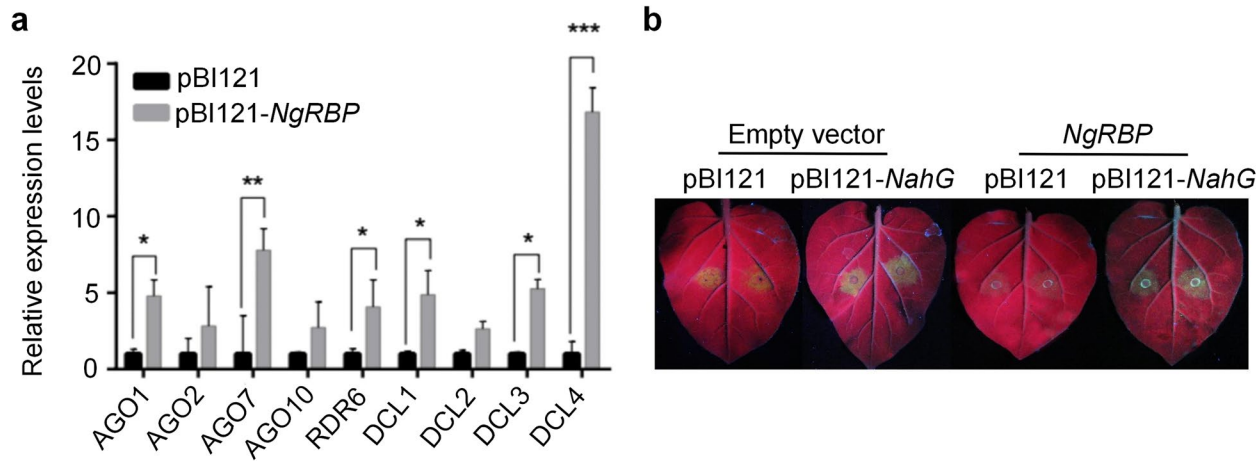

Fig. 7 RNA silencing was enhanced by *NgRBP* and hindered by *NahG* expression. **a** Expression analysis of RNA silencing pathway genes in the systemic leaves of plants with *NgRBP* transient expression and tobacco mosaic virus (TMV) infection. pBI121-*NgRBP* and the empty vector pBI121 were infiltrated into the leaves of *N. glutinosa,* respectively. One day later, TMV was inoculated in the above-infiltrated area, and the systemic leaves were sampled for gene expression analysis via RT-qPCR. The data were analyzed using Student's *t*-test, and asterisks denote significant differences between the two groups (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$). **b** Transient expression of *NahG* blocked RNA silencing enhancement mediated by *NgRBP.* First, pBI121-*NgRBP* and the empty vector pBI121 were infiltrated into the leaves of *N. glutinosa,* respectively. One day later, TMV was inoculated in the above-infiltrated area, and simultaneously, the systemic leaves were infiltrated with 35S-GFP and pBI121 or 35S-GFP and pBI121-*NahG,* respectively. GFP fluorescence was observed under UV light 3 d post inoculation of TMV

our discovery also explains why LAR mediated by TMV is effective not only against TMV but also against other viruses.

SAR can be activated by *R* gene-virus interactions, and it is effective against a wide range of pathogens, including viruses, bacteria, fungi, and oomycetes (Ryals et al. 1996, Sticher et al. 1997, Durrant & Dong 2004, Liu et al. 2010). The signal of SAR has gained particular attention due to its ability to transmit over long distances. Initially, both SA/MeSA and JA were thought to be SAR signals, but several studies have provided evidence to the contrary (Clarke et al. 2000, Truman et al. 2007, Attaran et al. 2009). Ethylene perception is also believed to be necessary for the generation of systemic signal molecules and SAR development (Verberne et al. 2003). SAR is characterized by increased expression of *PR* genes, although their roles in establishing SAR are unclear. SAR generally results from the concerted effects of many PR proteins rather than from a specific PR protein (van Loon & van Kammen 1970, Durrant & Dong 2004, Fu & Dong 2013). The activation of six groups of genes, including 1-aminocyclopropane-1-carboxylic acid oxidase gene (*ACCO*), 3-hydroxy-3-methylglutaryl-CoA reductase gene (*HMGR*), ascorbate peroxidase gene (*APX*), pathogenesis-related protein 3 gene (*PR3*), *SAR8.2e*, and proteinase-activated peceptor 1 gene (*PAR-1*), is associated with the development of SAR induced by TMV. Exogenous application of SA transiently induces these genes (Guo et al. 2000). However, the antiviral role of these genes remains unclear.

Although much progress has been made in SAR signalling, the exact mechanisms of SAR, particularly viral inhibition, are not yet completely understood. Our findings indicate that RNA silencing is activated by TMV-mediated HR and spreads to systemic tobacco leaves, playing a key role in viral resistance. In plants, RNA silencing is a crucial mechanism against viruses, and its systemic spread was initially demonstrated in grafting experiments (Yoo et al. 2004, Hewezi et al. 2005, Shaharuddin et al. 2006). Systemic silencing serves an antiviral function by initiating a resistant response in tissues where the virus has not yet infiltrated (Schwach et al. 2005). Obviously, RNA silencing exhibits systemic transmission and broad-spectrum antiviral properties, aligning with the characteristics of SAR. Studies have demonstrated that fewer viral siRNAs are produced when a virus infects transgenic plants with reduced SA accumulation, indicating that SA affects RNA silencing (Alamillo et al. 2006). A recent study showed that *Bacillus subtilis* 26D triggers SAR against *Rhopalosiphum padi* by regulating RNAi and microRNA pathways in wheat. This indicates that the connection between SAR and RNA silencing may be universal (Rumyantsev et al. 2023). To summarize, TMV-induced HR promoted local and systemic RNA silencing, and RNA silencing contributes to both LAR and SAR which are triggered against

viruses. These results would enrich the established HR and SAR models.

Conclusions

In this study, by using the *NgRBP* gene which confers stronger HR in *N. glutinosa* (NN), we found that a stronger HR led to intensified local and systemic RNA silencing, resulting in reduced virus accumulation in both local and systemic tissues. This observation suggests that RNA silencing plays a crucial role in inhibiting viruses during HR and SAR. Our findings uncover an intriguing interaction between *N* gene-mediated resistance and RNA silencing.

Methods

Plant materials and plasmid constructs

N. glutinosa plants were raised in a greenhouse at 24℃ under a 16 h light/8 h dark photoperiod. The full-length open reading frame (ORF) of 471-nt segment of *NgRBP* (Accession no. AF005359) was amplified from *N. glutinosa* total RNA via RT-PCR using high-fidelity DNA polymerase (TransGen, Beijing, China) (Huang et al. 2019). The PCR product was ligated to the pMD-19T vector and cloned into the binary vector pBI121 between the 35S promoter and the *Nos* terminator. The full-length sequence of *NgRBP*, except for the start codon, was inserted into TRV infectious clone to generate a VIGS vector. All fragments generated using PCR were confirmed via DNA sequencing. The recombinant plasmids were transformed using the freeze-thaw method into *Agrobacterium tumefaciens* strain GV3101, which contained the helper plasmid pJIC SA Rep (Höfgen & Willmitzer 1988).

Viral inoculation and detection

The *N. tabacum* leaves displaying TMV symptoms were homogenized in 0.1 M phosphate buffer (pH 7.2) using a sterile pestle and mortar, and the cell fragments were removed via centrifugation. Standardized volumes of infectious sap (or phosphate buffer as a control) were mechanically inoculated onto the upper surface of fully expanded *N. glutinosa* leaves sprayed with carborundum. The accumulation of virus in infected tissues was determined using reverse transcription (RT)-quantitative polymerase chain reaction (qPCR) for the TMV *CP* gene. Inoculated plants were grown in an insect-free greenhouse at 24℃, and the viral symptoms were monitored. Each experiment was replicated three times, including five independent plants.

Co-infiltration and GFP imaging

N. glutinosa plants at the five- or six-leaf stage were locally infiltrated with *Agrobacterium* GV3101, carrying the aforementioned constructs, using a 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) at a 1:1 (v/v) ratio before infiltration. Local and systemic RNA silencing was determined by observing GFP fluorescence in both 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).

Virus-induced gene silencing

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 with an ATG deletion was inserted into TRV2 to generate pTRV2:*NgRBP*. No homologous genes of *NgRBP* were found in *N. glutinosa* using National Center for Biotechnology Information (NCBI) BLAST. The pTRV1 and pTRV2:00 (empty vector) or pTRV2:*NgRBP* constructs were transformed into *Agrobacterium* GV3101. *Agrobacterium* cultures were resuspended in an infiltration buffer containing 10 mM $MgCl_2$, 10 mM MES, and 200 mM acetosyringone (pH 5.6; OD_{600} = 0.8). *Agrobacterium* cultures harbouring pTRV1 were mixed with pTRV2:00 or pTRV2:*NgRBP* in a 1:1 ratio and infiltrated into the lower leaves of 3-week-old *N. glutinosa* plants. The infiltrated plants were placed in the greenhouse at 24℃ and 70% RH under a long-day (16 h light/8 h dark) photoperiod. At 7 dpi, empty vector control, and *NgRBP*-silenced plants were used for *NgRBP* expression analyses and TMV inoculation assays.

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 the TIAN Script RT Kit (Tiangen, Beijing, China). RT-qPCR was performed using the Talent SYBR Green Kit (Tiangen, Beijing, China). The *Actin1* gene was used as an internal reference. Each reaction was conducted in triplicate and repeated three times. The results were analyzed using the Bio-Rad CFX Manager software (Bio-Rad, California, USA).

Total RNA and siRNA Northern blot analysis

As described previously, total and low-molecular-weight RNAs were extracted from the leaves (Jing et al. 2011). From each sample, 20 μg whole RNA aliquots were separated on 1% formaldehyde agarose gels and transferred to Hybond-N^+ membranes (GE Healthcare, Marlborough, MA, USA) by upward capillary transfer in 20× SSC buffer (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). The membranes were hybridized with digoxigenin (DIG)-labelled probes corresponding to the full-length ORFs of *GFP* and *NgRBP*, respectively. For siRNA detection, 15 μg low molecular weight RNAs were separated on a 5% polyacrylamide-7 M urea gel and transferred to a Hybond-N^+ membrane in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) 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 a DIG-labelled probe of *GFP* mRNA. Chemiluminescent detection was performed using a DIG Northern Starter Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions.

Abbreviations

BP	Biological process
CC	Cellular component
CMV	Cucumber mosaic virus
CP	Capsid protein
DEG	Differentially expressed gene
dpi	Days post-inoculation
GFP	Green fluorescent protein
GO	Gene ontology
hpi	Hours post inoculation
HR	Hypersensitive response
JA	Jasmonic acid
LAR	Local acquired resistance
MeSA	Methyl salicylate
MF	Molecular function
NO	Nitric oxide
NPR1	Nonexpressor of pathogenesis-related protein 1
ORF	Open reading frame
R gene	Resistance gene
ROS	Reactive oxygen species
RSS	RNA silencing suppressor
RT-qPCR	Reverse transcription-quantitative PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
TMV	Tobacco mosaic virus
TRV	Tobacco rattle virus
VIGS	Virus-induced gene silencing

Supplementary Information

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Additional file 1: Figure S1. Volcano maps of differentially expressed genes. **Figure S2.** Gene ontology (GO) enrichment histogram of differen tial genes.

Additional file 2: Table S1. Enrichment analysis of KEGG functions of differentially expressed genes. **Table S2.** Differentially expressed genes related to disease resistance according to RNA-seq.

Acknowledgments

Not applicable.

Authors' contributions

HL conceived and designed the experiments. RY and HL wrote the manuscript. WL and RY performed most of the experiments. HZ and HL conducted bioinformatics analysis. WL, CR, YZ, HM, and CZ participated in this research. All authors read and approved the final manuscript.

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Availability of data and materials

All data supporting the findings of this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

Abbink TEM, Tjernberg PA, Bol JF, Linthorst HJM. Tobacco mosaic virus helicase domain induces necrosis in N Gene-carrying tobacco in the absence of virus replication. Mol Plant Microbe Interact. 1998;11(12):1242–6. https://doi.org/10.1094/MPMI.1998.11.12.1242.

Alamillo JM, Saénz P, García JA. Salicylic acid-mediated and RNA-silencing defense mechanisms cooperate in the restriction of systemic spread of plum pox virus in tobacco. Plant J. 2006;48(2):217–27. https://doi.org/10.1111/j.1365-313X.2006.02861.x.

Allan AC, Lapidot M, Culver JN, Fluhr R. An early tobacco mosaic virus-induced oxidative burst in tobacco indicates extracellular perception of the virus coat protein. Plant Physiol. 2001;126(1):97–108. https://doi.org/10.1104/pp.126.1.97.

Attaran E, Zeier TE, Griebel T, Zeier J. Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. Plant Cell. 2009;21(3):954–71. https://doi.org/10.1105/tpc.108.063164.

Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. 1998;17(22):6739–46. https://doi.org/10.1093/emboj/17.22.6739.

Burch-Smith TM, Schiff M, Caplan JL, Tsao J, Czymmek K, Dinesh-Kumar SP. A novel role for the TIR domain in association with pathogen-derived elicitors. PLOS Biol. 2007;5(3):e68. https://doi.org/10.1371/journal.pbio.0050068.

Canto T, Palukaitis P. Novel N gene-associated, temperature-independent resistance to the movement of tobacco mosaic virus vectors neutralized by a cucumber mosaic virus RNA1 transgene. J Virol. 2002;76(24):12908–16. https://doi.org/10.1128/jvi.76.24.12908-12916.2002.

Carr JP, Lewsey MG, Palukaitis P. Chapter 3. In: Carr JP, Loebenstein G, editors. Advances in virus research (Vol. 76. p. 57–121). San Diego: Academic press; 2010.

Choudhary DK, Fau PA, Johri B. Induced systemic resistance (ISR) in plants: mechanism of action. Indian J Microbiol. 2007;47(4):289–97 (0046-8991 (Print)).
Chung E, Seong E, Kim YC, Chung EJ, Oh SK, Lee S, Park JM, Joung YH, Choi D. A method of high frequency virus-induced gene silencing in chili pepper (Capsicum annuum L. cv. Bukang). Mol Cells. 2004;17(2):377–80.
Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X. Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in *Arabidopsis*. Plant Cell. 2000;12(11):2175–90. https://doi.org/10.1105/tpc.12.11.2175.
Cole AB, Király L, Ross K, Schoelz JE. Uncoupling resistance from cell death in the hypersensitive response of *Nicotiana* species to cauliflower mosaic virus infection. Mol Plant Microbe Interact. 2001;14(1):31–41. https://doi.org/10.1094/MPMI.2001.14.1.31.
Darby RM, Maddison A, Mur LA, Bi YM, Draper J. Cell-specific expression of salicylate hydroxylase in an attempt to separate localized HR and systemic signalling establishing SAR in tobacco. Mol Plant Pathol. 2000;1(2):115–23. https://doi.org/10.1046/j.1364-3703.2000.00014.x.
del Pozo O, Lam E. Expression of the baculovirus p35 protein in tobacco affects cell death progression and compromises N gene-mediated disease resistance response to Tobacco mosaic virus. Mol Plant Microbe Interact. 2003;16(6):485–94. https://doi.org/10.1094/MPMI.2003.16.6.485.
Dunigan DD, Madlener JC. Serine/threonine protein phosphatase is required for tobacco mosaic virus-mediated programmed cell death. Virology. 1995;207(2):460–6. https://doi.org/10.1006/viro.1995.1105.
Durrant WE, Dong X. Systemic acquired resistance. Annu Rev Phytopathol. 2004;42:185–209. https://doi.org/10.1146/annurev.phyto.42.040803.140421.
Fodor J, Hideg E, Kecskés A, Király Z. In vivo detection of tobacco mosaic virus-induced local and systemic oxidative burst by electron paramagnetic resonance spectroscopy. Plant Cell Physiol. 2001;42(7):775–9. https://doi.org/10.1093/pcp/pce096.
Fu ZQ, Dong X. Systemic acquired resistance: turning local infection into global defence. Annu Rev Plant Biol. 2013;64:839–63. https://doi.org/10.1146/annurev-arplant-042811-105606.
Guo A, Salih G, Klessig DF. Activation of a diverse set of genes during the tobacco resistance response to TMV is independent of salicylic acid; induction of a subset is also ethylene independent. Plant J. 2000;21(5):409–18. https://doi.org/10.1046/j.1365-313x.2000.00692.x.
Hewezi T, Alibert G, Kallerhoff J. Local infiltration of high- and low-molecular-weight RNA from silenced sunflower (*Helianthus annuus* L.) plants triggers post-transcriptional gene silencing in non-silenced plants. Plant Biotechnol J. 2005;3(1):81–9. https://doi.org/10.1111/j.1467-7652.2004.00103.x.
Höfgen R, Willmitzer L. Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res. 1988;16(20):9877. https://doi.org/10.1093/nar/16.20.9877.
Huang X, Yu R, Li W, Geng L, Jing X, Zhu C, Liu H. Identification and characterisation of a glycine-rich RNA-binding protein as an endogenous suppressor of RNA silencing from *Nicotiana glutinosa*. Planta. 2019;249(6):1811–22. https://doi.org/10.1007/s00425-019-03122-5.
Jacques A, Ghannam A, Erhardt M, de Ruffray P, Baillieul F, Kauffmann S. NtLRP1, a tobacco leucine-rich repeat gene with a possible role as a modulator of the hypersensitive response. Mol Plant Microbe Interact. 2006;19(7):747–57. https://doi.org/10.1094/MPMI-19-0747.
Jing XL, Fan MN, Jia G, Liu LW, Ma L, Zheng CC, Zhu XP, Liu HM, Wang XY. A multifunctional protein encoded by turkey herpesvirus suppresses RNA silencing in *Nicotiana benthamiana*. J Virol. 2011;85(23):12792–803. https://doi.org/10.1128/JVI.05565-11.
Jones JDG, Dangl JL. The plant immune system. Nature. 2006;444(7117):323–9. https://doi.org/10.1038/nature05286.
Kang BC, Yeam I, Jahn MM. Genetics of plant virus resistance. Annu Rev Phytopathol. 2005;43:581–621. https://doi.org/10.1146/annurev.phyto.43.011205.141140.
Klessig DF, Choi HW, Dempsey DA. Systemic acquired resistance and salicylic acid: past, present, and future. Mol Plant Microbe Interact. 2018;31(9):871–88. https://doi.org/10.1094/MPMI-03-18-0067-CR.
Lacomme C, Santa Cruz S. Bax-induced cell death in tobacco is similar to the hypersensitive response. Proc Natl Acad Sci U S A. 1999;96(14):7956–61. https://doi.org/10.1073/pnas.96.14.7956.
Liu PP, Bhattacharjee S, Klessig DF, Moffett P. Systemic acquired resistance is induced by R gene-mediated responses independent of cell death. Mol Plant Pathol. 2010;11(1):155–60. https://doi.org/10.1111/j.1364-3703.2009.00564.x.
Loebenstein G, Gera A. Inhibitor of virus replication released from tobacco mosaic virus-infected protoplasts of a local lesion-responding tobacco cultivar. Virology. 1981;114(1):132–9. https://doi.org/10.1016/0042-6822(81)90259-2.
Loebenstein G, Gera A, Barnett A, Shabtai S, Cohen J. Effect of 2,4-dichlorophenoxyacetic acid on multiplication of tobacco mosaic virus in protoplasts from local-lesion and systemic-responding tobaccos. Virology. 1980;100(1):110–5. https://doi.org/10.1016/0042-6822(80)90556-5.
Luna E, Ton J. The epigenetic machinery controlling transgenerational systemic acquired resistance. Plant Signal Behav. 2012;7(6):615–8. https://doi.org/10.4161/psb.20155.
Malamy J, Carr JP, Klessig DF, Raskin I. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science. 1990;250(4983):1002–4. https://doi.org/10.1126/science.250.4983.1002.
Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science. 1990;250(4983):1004–6. https://doi.org/10.1126/science.250.4983.1004.
Mur LA, Kenton P, Draper J. Something in the air: volatile signals in plant defence. Trends Microbiol. 1997;5(8):297–300. https://doi.org/10.1016/S0966-842X(97)01097-4.
Mur LAJ, Carver TLW, Prats E. NO way to live; the various roles of nitric oxide in plant-pathogen interactions. J Exp Bot. 2006;57(3):489–505. https://doi.org/10.1093/jxb/erj052.
Murphy AM, Gilliland A, Eng Wong C, West J, Singh DP, Carr JP. Signal transduction in resistance to plant viruses. Eur J Plant Pathol. 2001;107(1):121–8. https://doi.org/10.1023/A:1008732123834.
Pruss GJ, Lawrence CB, Bass T, Li QQ, Bowman LH, Vance V. The potyviral suppressor of RNA silencing confers enhanced resistance to multiple pathogens. Virology. 2004;320(1):107–20. https://doi.org/10.1016/j.virol.2003.11.027.
Rumyantsev SD, Veselova SV, Burkhanova GF, Alekseev VY, Maksimov IV. Bacillus subtilis 26D triggers induced systemic resistance against *Rhopalosiphum padi* L. by regulating the expression of genes AGO, DCL and microRNA in bread spring wheat. Microorganisms 2023;11(12):2983. https://doi.org/10.3390/microorganisms11122983.
Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD. Systemic acquired resistance. Plant Cell. 1996;8(10):1809–19. https://doi.org/10.1105/tpc.8.10.1809.
Schwach F, Vaistij FE, Jones L, Baulcombe DC. An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol. 2005;138(4):1842–52.
Shaharuddin NA, Han Y, Li H, Grierson D. The mechanism of graft transmission of sense and antisense gene silencing in tomato plants. FEBS Lett. 2006;580(28–29):6579–86. https://doi.org/10.1016/j.febslet.2006.11.005.
Shimomura T, Dijkstra J. The occurrence of callose during the process of local lesion formation. Neth J Plant Pathol. 1975;81(3):107–21. https://doi.org/10.1007/BF01999861.
Silhavy D, Molnár A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyán J. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. 2002;21(12):3070–80. https://doi.org/10.1093/emboj/cdf312.
Sticher L, Mauch-Mani B, Métraux JP. Systemic acquired resistance. Annu Rev Phytopathol. 1997;35:235–70. https://doi.org/10.1146/annurev.phyto.35.1.235. PMID: 15012523.
Thomma BP, Eggermont K, Penninckx IA, Mauch-Mani B, Vogelsang R, Cammue BP, Broekaert WF. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci U S A. 1998;95(25):15107–11. https://doi.org/10.1073/pnas.95.25.15107.
Truman W, Bennett MH, Kubigsteltig I, Turnbull C, Grant M. *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. Proc Natl Acad Sci U S A. 2007;104(3):1075–80. https://doi.org/10.1073/pnas.0605423104.
van Loon LC, van Kammen A. Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun *NN*". II. Changes in protein constitution after infection with tobacco

mosaic virus. Virology. 1970;40(2):190–211. https://doi.org/10.1016/0042-6822(700)9395-8.
Verberne MC, Hoekstra J, Bol JF, Linthorst HJM. Signaling of systemic acquired resistance in tobacco depends on ethylene perception. Plant J. 2003;35(1):27–32. https://doi.org/10.1046/j.1365-313x.2003.01778.x.
Vlot AC, Dempsey DA, Klessig DF. Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol. 2009;47:177–206. https://doi.org/10.1146/annurev.phyto.050908.135202.
Watt LG, Crawshaw S, Rhee SJ, Murphy AM, Canto T, Carr JP. The cucumber mosaic virus 1a protein regulates interactions between the 2b protein and Argonaute 1 while maintaining the silencing suppressor activity of the 2b protein. PLOS Pathog. 2020;16(12):e1009125. https://doi.org/10.1371/journal.ppat.1009125.
Weintraub M, Ragetli HW. An electron microscope study of tobacco mosaic virus lesions in Nicotiana glutinosa l. J Cell Biol. 1964;23(3):499–509. https://doi.org/10.1083/jcb.23.3.499.
Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. Cell. 1994;78(6):1101–15. https://doi.org/10.1016/0092-8674(94)90283-6.
Wright KM, Duncan GH, Pradel KS, Carr F, Wood S, Oparka KJ, Cruz SS. Analysis of the N gene hypersensitive response induced by a fluorescently tagged tobacco mosaic virus. Plant Physiol. 2000;123(4):1375–86. https://doi.org/10.1104/pp.123.4.1375.
Wu JH, Dimitman JE. Leaf structure and callose formation as determinants of TMV movement in bean leaves as revealed by UV irradiation studies. Virology. 1970;40(4):820–7. https://doi.org/10.1016/0042-6822(70)90127-3.
Yamamoto S, Katagiri M, Maeno H, Hayaishi O. Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and general properties. J Biol Chem. 1965;240:3408–13.
Yoo BC, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee YM, Lough TJ, Lucas WJ. A systemic small RNA signaling system in plants. Plant Cell. 2004;16(8):1979–2000. https://doi.org/10.1105/tpc.104.023614.
Zhang Y, Song G, Lal NK, Nagalakshmi U, Li Y, Zheng W, Huang PJ, Branon TC, Ting AY, Walley JW, Dinesh-Kumar SP. TurboID-based proximity labeling reveals that UBR7 is a regulator of N NLR immune receptor-mediated immunity. Nat Commun. 2019;10(1):3252. https://doi.org/10.1038/s41467-019-11202-z.