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Small RNA derived from *Tobacco mosaic virus* targets a host C2-domain abscisic acid-related (CAR) 7-like protein gene

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Abstract

Tobacco mosaic virus (TMV) is a positive-sense single-stranded RNA virus. The 3' end of TMV genome is consisted of an upstream pseudoknot domain (UPD) and a tRNA-like structure (TLS), both of which are important RNA elements to enhance TMV replication and translation. Deep-sequencing analysis revealed that TMV-specific viral small interfering RNAs (vsRNAs) were generated in TMV-infected *Nicotiana benthamiana* plants. A vsRNA derived from the juxtaposition between UPD and TLS, named TMV-vsRNA 22 nt (6285–6306), possessed high sequence complementarity to a host gene which encodes a C2-domain abscisic acid (ABA)-related (CAR) 7-like protein. CAR proteins play a critical role in ABA signaling pathway. The CAR protein-encoding gene was amplified from *N. benthamiana* leaves and termed as *Nb-CAR7*. In TMV-infected plants, accumulation of *Nb-CAR7* transcripts was significantly decreased, as compared with that of mock-inoculated and TMV-43A-infected plants. TMV-43A is a mutant without the UPD sequence in its genome. Overexpression of *Nb-CAR7* led to decreased TMV RNA accumulation in the TMV-inoculated leaves. Silencing of *Nb-CAR7* enhanced TMV replication and resulted in a higher viral RNA accumulation. In addition, the expression level of *Nb-CAR7* was positively correlated to that of a low-temperature-induced ABA responsive gene (*LTI65*). The effect of *Nb-CAR7* on TMV RNA accumulation in host plants was linked to ABA signaling pathway. In conclusion, a vsRNA derived from the juxtaposition between UPD and TLS at the 3'UTR of TMV targets a host *CAR7* gene.

Keywords: *Tobacco mosaic virus*, Virus small interfering RNA, C2 domain ABA-related (CAR) gene

Background

Tobacco mosaic virus (TMV) is a positive-sense single-stranded RNA virus in genus *Tobamovirus*. It has a wide host range and its infection can cause severe damages and yield losses in many economically important crops (Scholthof et al. 2011). In its natural host tobacco and other closely related species, TMV can induce chlorosis, mosaic and necrosis in plant tissues. The virus is mechanically transmitted, resulting in quick and effective infection (Scholthof 2004). Discovery of new molecular interactions

between TMV and its host proteins will add to our knowledge to connect missing links and help in developing control strategies to prevent spread of the virus.

The RNA genome of TMV contains four open reading frames that encode essential viral proteins for replication, movement and encapsidation. There are also several elements involved in control of replication and translation of viral genes in the 5' and 3' untranslated regions (UTRs). For example, the upstream pseudoknot domain (UPD) and tRNA-like structure (TLS) in the 3' UTR play important roles in virus replication (Gallie and Walbot 1990; Takamatsu et al. 1990). The TLS of TMV can be aminoacylated and some primary RNA elements in UPD interact with host plant elongation factors during virus replication (Gallie et al. 1991; Osman et al.

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2000; Zeenko et al. 2002). A novel stem-loop structure located upstream of the UPD of TMV has been identified to regulate virus replication. Disruption of this stem-loop structure enhances TMV infectivity and viral RNA accumulation (Guo and Wong 2018).

RNA silencing is an evolutionarily conserved mechanism in eukaryotes. It is a defense mechanism induced by double-stranded RNAs (dsRNAs) or hairpin structured RNA (hpRNA) that leads to degradation of target RNAs (Zhang et al. 2019). In plants, RNA silencing acts as a defense system against infection by pathogens, including fungi, bacteria, viruses or viroids, which results in the production of pathogen-specific short interfering RNAs (siRNAs) (Guo et al. 2016). In virus-infected plants, virus-derived small interfering RNAs (vsiRNAs) are produced from double-stranded viral RNA by host plant enzymes. The vsiRNAs are loaded into Argonaute (AGO)-containing complexes known as RNA-induced silencing complexes (RISCs), to promote the degradation of both genomic and subgenomic viral RNAs in a sequence-specific manner (Eamens et al. 2008).

The accumulation of vsiRNAs is an indication of the presence of RNA silencing in plants after virus infection (Li et al. 2018). In order to overcome the host antiviral RNA silencing system, plant viruses encode RNAi-suppressors and use them to counter the host RNA-factors and to weaken host defense. Most suppressors bind vsiRNAs to prevent degradation of viral RNAs so as to inhibit RNA silencing effects (Bivalkar-Mehla et al. 2011). Some suppressors interact with host genes to compromise antiviral functions of these genes (Li and Wang 2018). Moreover, post-transcriptional gene silencing is compromised in the presence of viral RNAi suppressors, which leads to over-expression of host defense-related genes to protect the host against viral infection (Pumplin and Voinnet 2013). For example, in *Sugarcane mosaic virus* (SCMV)-infected maize, the binding of RNAi-suppressor of SCMV (HC-Pro) with the violaxanthin deep oxidase protein of maize (ZmVDE) down-regulates the RNA silencing suppression activity of HC-Pro and results in reduced accumulation of viral RNA and coat protein in the infected cells (Chen et al. 2017).

A chimeric virus TMV-43A was created previously (Niu et al. 2015). In TMV-43A genome, the original TMV-UPD sequence is deleted and substituted by a 43-nt poly(A) tract. TMV-43A induces mosaic symptoms in *N. benthamiana* and replicates slower than TMV (Niu et al. 2015). Comparing with the vsiRNAs generated in TMV-43A-infected plants, many TMV UPD-specific vsiRNAs were generated in TMV-infected plants. Either partial or entire sequences of these TMV-specific vsiRNAs were all mapped to TMV-UPD. These vsiRNAs are predicted to target a number of host genes involved in host responses and various signaling pathways (Guo and Wong 2017). Since TMV-43A does not contain the UPD sequence, it can't generate vsiRNAs with UPD sequence.

Abscisis acid (ABA) is a key hormone involved in plant responses to abiotic stresses and has great impacts on plant defense against various pathogens (Alazem and Lin 2015). For example, ABA induces different plant resistance responses to viruses to interfere with virus accumulation (Alazem and Lin 2017); ABA induces callose deposition at plasmodesmata, a mechanism that limits viral cell-to-cell movement and reduces virus spread throughout the whole plant (Mauch-Mani and Mauch 2005). A number of proteins are involved in ABA biosynthesis and signaling pathway in plants. The ten-member family of C2-domain ABA-related (CAR) proteins in *Arabidopsis thaliana* consists of single C2 domains that interact with pyrabactin resistance 1/PYR1-like (PYR/PYL) ABA receptors (Rodriguez et al. 2014). Structural studies revealed that CAR proteins have two lipid binding sites which are calcium (Ca^{2+}) sensors with a basal phospholipid binding activity. Clusters of CAR proteins act as a membrane assembly site for ABA receptors through Ca^{2+} -dependent recruitment. This process is relevant to ABA signaling and CARs represent a signaling node that connects the ABA and Ca^{2+} pathways to the membrane (Diaz et al. 2016).

In this study, we discovered a vsiRNA derived from the juxtaposition of TMV-UPD and TLS. It has near-perfect complementarity with the CAR 7-like protein-encoding gene in *N. benthamiana* plants, termed as *Nb-CAR7*. This vsiRNA directly silenced the *Nb-CAR7* gene during viral infection. Accompanying with a high accumulation of TMV-vsiRNA in plants, the expression level of *Nb-CAR7* was significantly decreased in TMV-infected plants, as compared to that of mock-inoculated and TMV-43A-infected plants. Transient overexpression of *Nb-CAR7* inhibited TMV infection and resulted in reduced amount of viral RNAs in infiltrated leaves. Silencing of *Nb-CAR7* in *N. benthamiana* plants enhanced TMV replication and led to a higher accumulation of viral RNAs. Moreover, the expression of *Nb-CAR7* was correlated to that of a low-temperature-induced 65 KDa protein (LTI65) gene and also an ABA marker gene. Therefore, it is deduced that the effect of *Nb-CAR7* on TMV RNA accumulation was linked to ABA signaling pathway. In summary, our findings showed that a vsiRNA derived from the juxtaposition between UPD and TLS in the 3' UTR of TMV targets a host *CAR7* gene in *N. benthamiana* plants.

Results

vsiRNA derived from the UPD region of TMV genome was detected in TMV-infected *N. benthamiana* plants

Based on our previously published results on small RNA sequencing of TMV-infected *N. benthamiana* plants, a total of 32,369 siRNA reads were mapped to TMV genome (Guo and Wong 2017). The vsiRNAs sequences (either partial or full) mapped to TMV-UPD were selected for analysis. The vsiRNA generated from nucleotides (nt) 6285 to 6306 in TMV positive-sense strand showed the

highest read counts and was termed as TMV-vsiRNA 22 nt (6285–6306) (Fig. 1a). As the TMV-UPD region expands from 6192 to 6288 nt, TMV-vsiRNA 22 nt (6285–6306) was mapped to the juxtaposition encompassing the UPD and TLS (Fig. 1b). As TMV-43A does not contain the UPD sequence, the TMV-vsiRNA 22 nt (6285–6306) is not generated in TMV-43A infected plants.

The presence of TMV-vsiRNA 22 nt (6285–6306) was validated through Northern blot. High accumulation of TMV-vsiRNA 22 nt (6285–6306) was detected in TMV-infected *N. benthamiana* plants at 4 days post inoculation (dpi) (Fig. 1c). We compared TMV-vsiRNA 22 nt (6285–6306) sequence with small RNAs generated from TMV-43A-infected *N. benthamiana* (Guo and Wong 2017) and found no match between them. This is due to the reason that TMV-43A does not possess the UPD sequence in its viral genome.

Validation of the putative target gene of TMV-vsiRNA 22 nt (6285–6306) encoding a C2-domain ABA-related 7-like protein in *N. benthamiana* plants

To identify the potential target genes of TMV-vsiRNA 22 nt (6285–6306) in host plant, the TMV-vsiRNA 22 nt (6285–

6306) sequence was aligned to *N. benthamiana* draft genome sequence using an online plant small RNA target analysis tool psRNATarget (Dai et al. 2018). A transcript sequence located in *N. benthamiana* genome scaffold Niben101Scf12415g00017.1 showed near-perfect sequence complementarity with TMV-vsiRNA 22 nt (6285–6306) (Fig. 2a). The BLASTn results showed that this transcript shared 97% (491/504) sequence identity with a predicted *N. benthamiana* C2-domain ABA-related 7-like gene termed as *Nb-CAR7*. The accumulation of *Nb-CAR7* transcript in TMV-infected *N. benthamiana* plants was significantly lower than that in mock-inoculated plants at 4 dpi. The expression level of *Nb-CAR7* in the TMV-43A-infected plants remained the same as that in the mock-inoculated plants (Fig. 2b).

The mRNA cleavage of *Nb-CAR7* by TMV-vsiRNA 22 nt (6285–6306) was validated through 5'-RNA-linker-mediated RACE (5'-RLM-RACE). A target DNA fragment was successfully amplified from TMV-infected *N. benthamiana* leaves, but this product was not amplified from mock-inoculated plants (Fig. 2c), indicating the specificity of TMV-vsiRNA 22 nt-directed cleavage of the *Nb-CAR7* transcript in TMV-infected *N. benthamiana* plants.

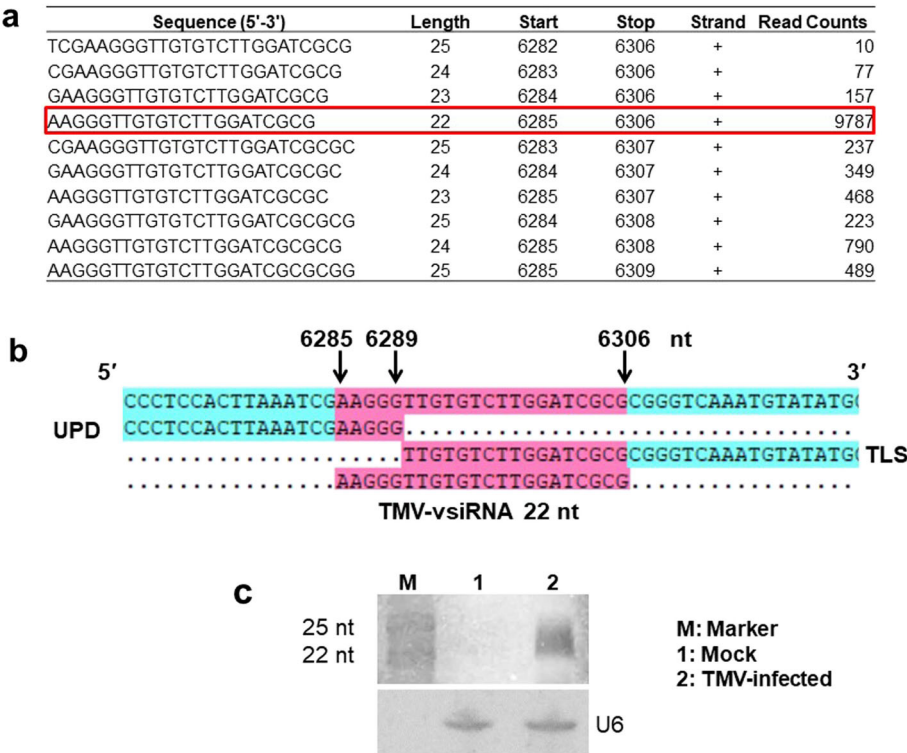


Fig. 1 vsiRNA derived from the UPD region of TMV genome was detected in TMV-infected *N. benthamiana* plants. **a** vsiRNA generated from nt 6285 to nt 6306 in TMV positive-sense strand was termed as TMV-vsiRNA 22 nt (6285–6306). It showed the highest read counts of 9787 (read box) in all TMV-UPD-vsiRNAs. **b** The exact nucleotide positions of TMV-vsiRNA 22 nt (6285–6306) sequence, which was mapped to the juxtaposition of UPD and TLS. **c** Northern blot analysis showed that TMV-vsiRNA 22 nt (6285–6306) was detected in TMV-infected *N. benthamiana* plants at 4 dpi but not in mock plants. U6 stands for U6 snRNA used as loading control

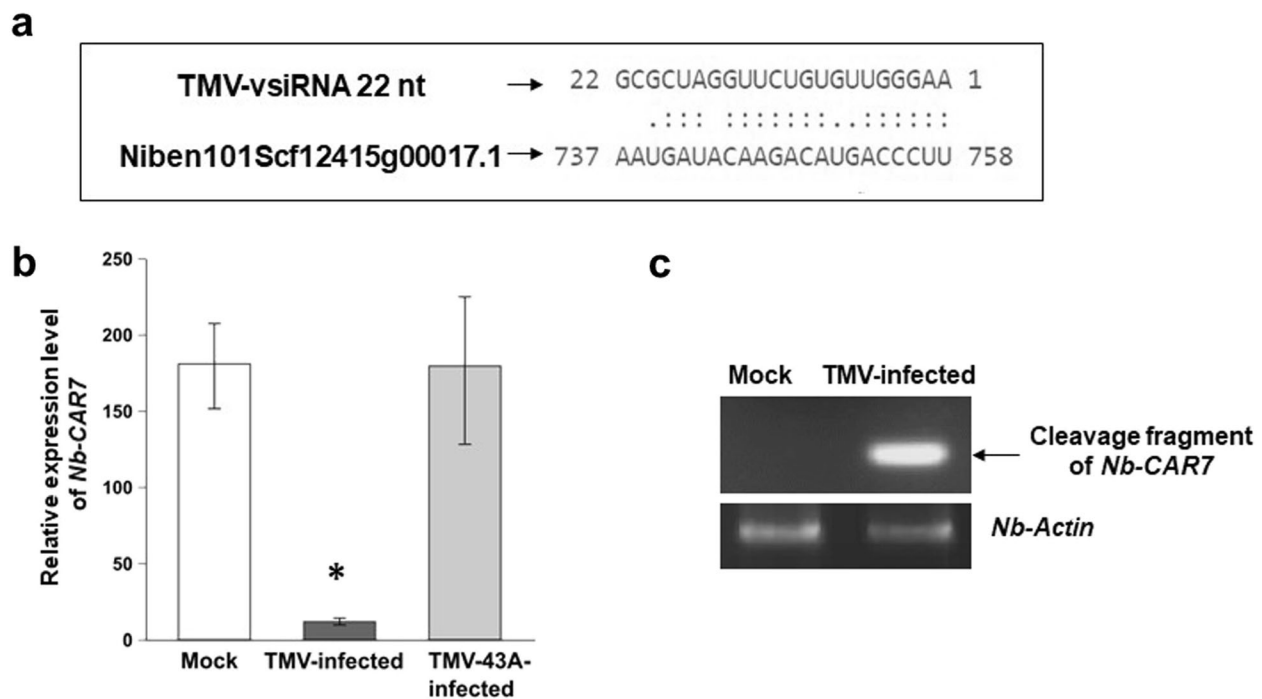


Fig. 2 TMV-vsiRNA 22 nt (6285–6306) targets a C2-domain ABA-related 7-like protein in *N. benthamiana* plants after virus infection. **a** Prediction of a putative host target gene of TMV-vsiRNA 22 nt (6285–6306). A transcript sequence located in *N. benthamiana* genome scaffold Niben101Scf12415g00017.1 showed near-perfect sequence complementarity with TMV-vsiRNA 22 nt (6285–6306). **b** The relative expression level of Nb-CAR7 in TMV-infected plants was lower than that in mock-inoculated and TMV-43A-infected *N. benthamiana* plants at 4 dpi. * represents $P < 0.05$ compared with mock-inoculated group. **c** A unique Nb-CAR7 fragment was amplified by 5'-RLM-RACE from the TMV-infected *N. benthamiana* plants but not from the mock-inoculated plants, indicating TMV-vsiRNA 22 nt-directed cleavage of the Nb-CAR7 transcript

Transient overexpression of Nb-CAR7 inhibited TMV RNA accumulation in *N. benthamiana* plants

To examine the effect of Nb-CAR7 on TMV infection, Nb-CAR7 overexpression construct driven by the 35S promoter with GFP tag was agro-infiltrated into *N. benthamiana* leaves (Fig. 3a). At 3 days after infiltration, the expression level of Nb-CAR7 was significantly increased in the 35S-NbCAR7-GFP-infiltrated leaves, as compared to that of GFP-expressed control plants (Fig. 3b). After TMV was inoculated onto the infiltrated leaves, TMV RNA accumulation was significantly reduced in the inoculated leaves of Nb-CAR7-overexpressed plants at 7 dpi, whereas was higher in the upper leaves of the same plant, as compared with that of the control plants (Fig. 3c).

After TMV inoculation, the transcript levels of Nb-CAR7 in the inoculated leaves and upper leaves of infiltrated plants at 7 dpi were also analysed (Fig. 3d). The transcript level of Nb-CAR7 in the TMV-inoculated leaves was higher than that of control plants. Conversely, Nb-CAR7 level in the upper leaves was lower than that of control.

Silencing of Nb-CAR7 enhanced TMV RNA accumulation in *N. benthamiana* plants

To examine the roles of Nb-CAR7 in TMV infection, *N. benthamiana* plants were infiltrated first with a Tobacco

rattle virus (TRV) vector containing partial Nb-CAR7 sequence (101–497 nt) (TRV2:Nb-CAR7) to silence the expression of Nb-CAR7. At 7 days post infiltration, transcription of Nb-CAR7 was not detected in the infiltrated leaves, indicating that silencing of Nb-CAR7 had occurred. The transcript level of Nb-CAR7 decreased in the upper leaves of silenced plants, as compared to that of TRV2-infiltrated control plants (Fig. 4a). While the expression of Nb-CAR7 was not detected after TRV-induced silencing, TMV RNA accumulation was increased in both the TMV-inoculated and upper leaves of the Nb-CAR7-silenced plants (Fig. 4b). These results suggested that silencing of Nb-CAR7 enhanced TMV RNA accumulation in *N. benthamiana* plants.

Effect of Nb-CAR7 on TMV RNA accumulation was linked to ABA signaling pathway

CAR proteins play important roles in plant ABA signaling pathway. *LTI65* is an ABA marker gene with an ABA-induced cis-acting element in its promoter region (Yamaguchi-Shinozaki and Shinozaki 1994). Our results showed that the relative expression level of Nb-LTI65 was up-regulated in transiently Nb-CAR7-overexpressed leaves of *N. benthamiana* plants after TMV infection (Fig. 5a) but was down-regulated in Nb-CAR7-silenced

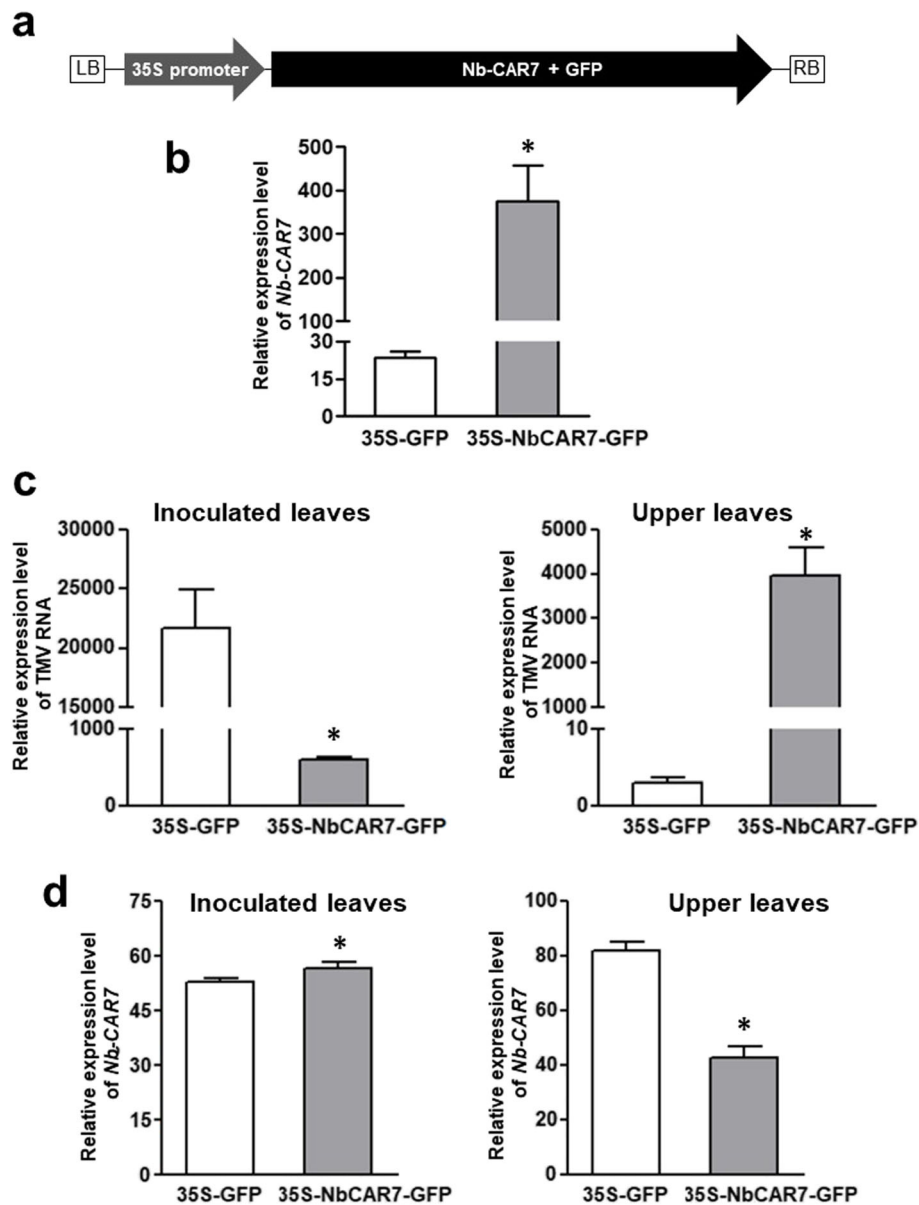


Fig. 3 Transient overexpression of *Nb-CAR7* inhibited TMV RNA accumulation in *N. benthamiana* plants. **a** Schematic structure of *Nb-CAR7* overexpression vector, 35S-NbCAR7-GFP. **b** The relative expression level of *Nb-CAR7* was increased in plant leaves at 3 days post infiltration with 35S-NbCAR7-GFP vector, using plants infiltrated with 35S-GFP vector as controls. **c** After TMV inoculation of the plants pre-infiltrated with 35S-NbCAR7-GFP vector, the relative expression level of TMV RNA at 7 dpi was decreased in the TMV-inoculated leaves but increased in the upper leaves. **d** After TMV inoculation of the plants pre-infiltrated with 35S-NbCAR7-GFP vector, the relative expression level of *Nb-CAR7* in the TMV-inoculated leaves was higher than that of control plants at 7 dpi, whereas was lower in the upper leaves than that of control plants. * represents $P < 0.05$ compared with control group

plants (Fig. 5b). Therefore, the effect of *Nb-CAR7* on TMV RNA accumulation was linked to the ABA signaling pathway.

Discussion

Small RNA-mediated silencing is a widespread antiviral mechanism in plants and other organisms. Many viruses encode suppressors of RNA silencing to counter defense.

The p126 protein of TMV is an RNA silencing suppressor and multiple domains of p126 protein can independently suppress local and systemic RNA silencing (Wang et al. 2012). The vsiRNAs have been reported to target host genes to mediate disease symptoms in plants. Those generated from *Cucumber mosaic virus* (CMV) Y-satellite (Y-sat) RNA directed gene silencing of host chlorophyll biosynthesis-related gene and induced chlorotic symptom in plants after virus infection

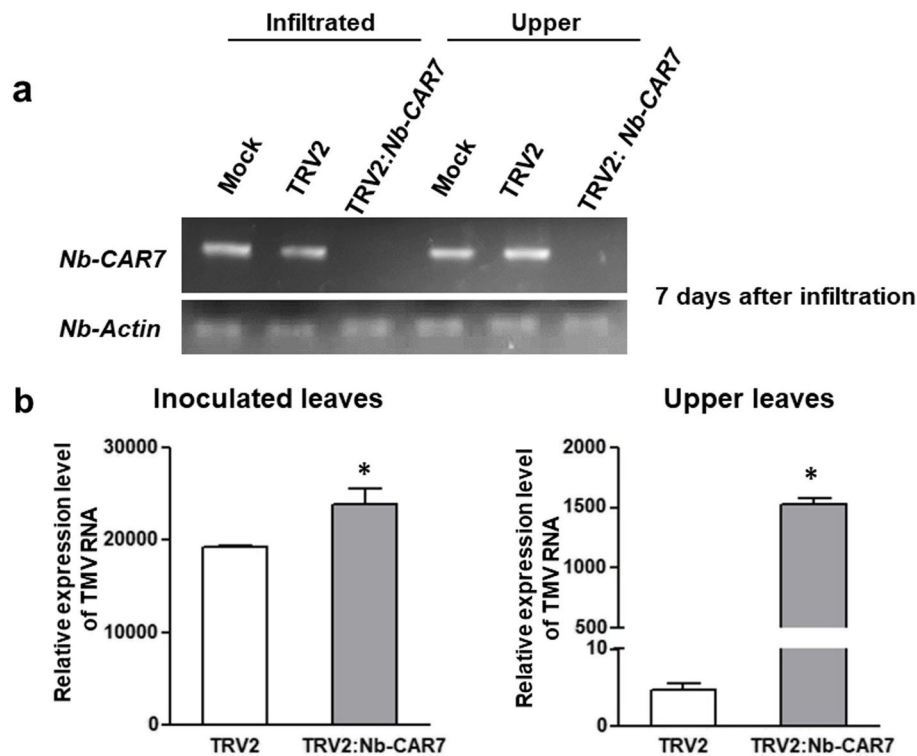


Fig. 4 Silencing of *Nb-CAR7* enhanced TMV RNA accumulation in *N. benthamiana* plants. **a** The *Nb-CAR7* transcript was not detected in both the infiltrated leaves and upper leaves of the TRV2:*Nb-CAR7*-infiltrated plants at 7 days post infiltration. As a control, the *Nb-Actin* was detected in the same plants at the same time point. **b** Relative accumulation of TMV RNA was increased in both TMV-inoculated and upper leaves of the *Nb-CAR7*-silenced plants. * represents $P < 0.05$ compared with control group

(Shimura et al. 2011). In this study, the TMV-derived vsiRNAs showed sequence complementarity to the host gene *Nb-CAR7* that belongs to the ten-member family of CAR proteins in plants. CAR proteins act as a membrane assembly site for ABA receptors (Diaz et al. 2016). Transient calcium-dependent interactions of PYR/PYL ABA receptors with membranes are mediated through CAR proteins, indicating the importance of CAR proteins in early ABA signaling processes (Rodriguez et al. 2014). After TMV infection, the accumulation of TMV-vsiRNA 22 nt (6285–6306) resulted in silencing of the *Nb-CAR7* gene. When *Nb-CAR7* was overexpressed, TMV RNA accumulation was reduced in host plants. On the other hand, when *Nb-CAR7* was silenced in plants, TMV RNA accumulation increased. These results showed a negative correlation between transcript abundance of *Nb-CAR7* and TMV RNA accumulation.

All CAR proteins contain the C2 domain, which is a Ca^{2+} -dependent membrane-targeting module present in many cellular proteins that are involved in signal transduction or membrane trafficking. The unique feature of C2 domain is that it has a wide range of lipid selectivity for major components of cell membranes. In addition, it is involved in Ca^{2+} -dependent phospholipid binding and in membrane targeting which guides lipids to the correct locations of ABA receptors (Stahelin et al. 2003; Corbin

et al. 2007; Rodriguez et al. 2014; Diaz et al. 2016). Therefore, CAR proteins play a critical role in the ABA signaling process.

The nucleotide sequence of *Nb-CAR7* was compared with those of other *CAR7* genes in tobacco plants and the result (Fig. 6) showed that *CAR7* genes are conserved among the tobacco plants, and these genes showed a high similarity with each other. The TMV-vsiRNA 22 nt (6285–6306) would also be able to target *CAR7* genes in other tobacco plants, to counterwork host defense responses after virus infection.

The expression of *LTI65* is induced in response to water deprivation conditions such as cold, high salt, and desiccation. The responses are mediated through ABA-dependent signaling pathway (Yamaguchi-Shinozaki and Shinozaki 1993; Uno et al. 2000; Nakashima et al. 2006). In this study, *Nb-LTI65* was amplified and used as a marker gene to monitor the level of ABA expression in transiently *Nb-CAR7*-overexpressed leaves or TRV-induced-*Nb-CAR7*-silenced plants after TMV infection. The transcript level of *Nb-LTI65* was increased in *Nb-CAR7*-overexpressed leaves, and decreased in *Nb-CAR7*-silenced plants, suggesting that ABA expression varied in accordance with the amount of *Nb-CAR7* in plants. Our results showed a positive correlation between the expression of *Nb-CAR7* and *Nb-LTI65*. However, there

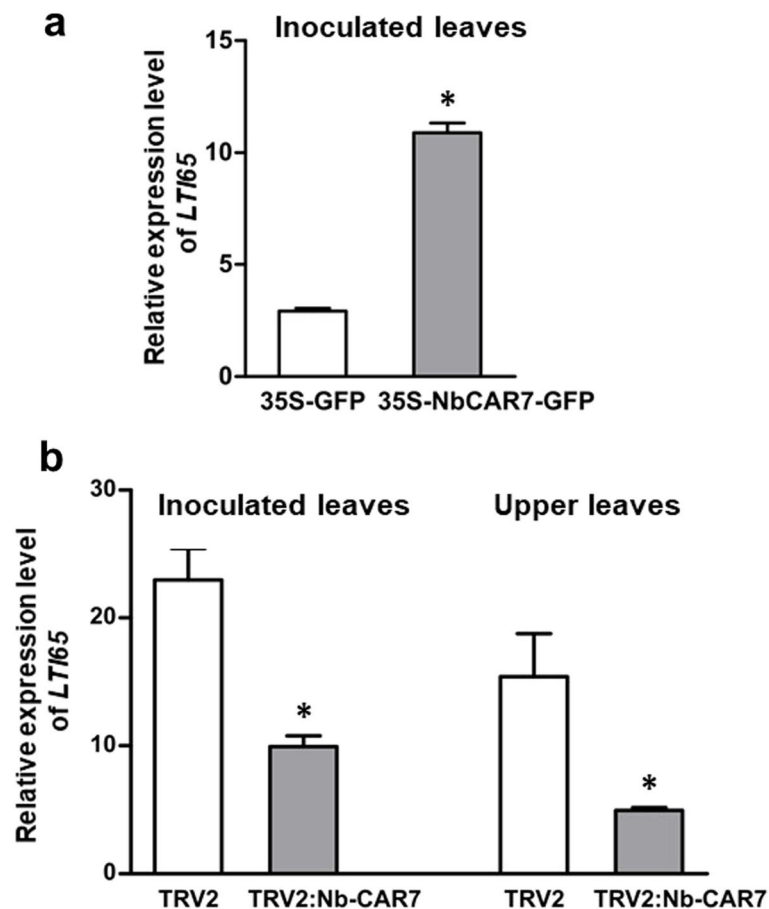


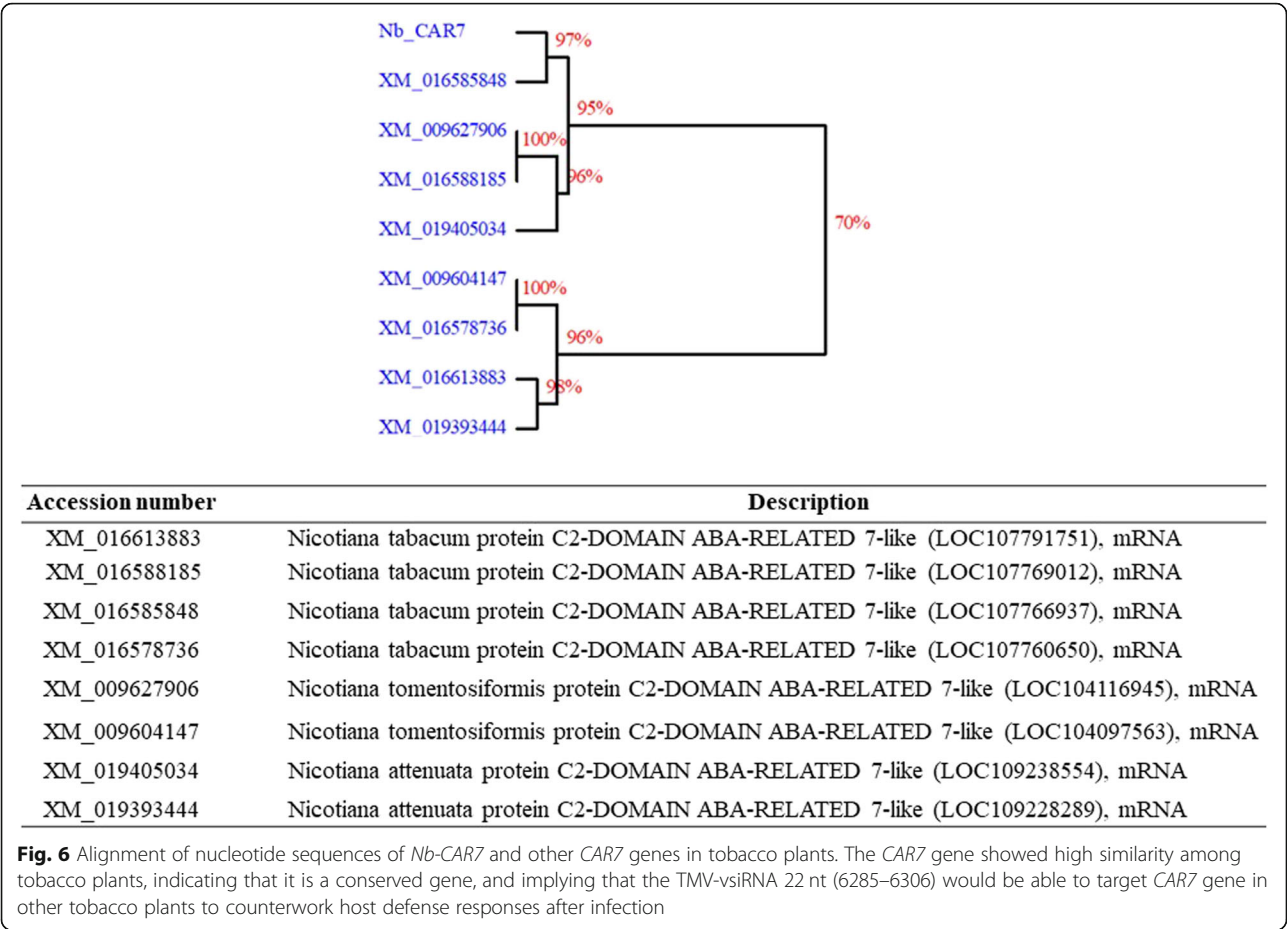
Fig. 5 Effect of *Nb-CAR7* on TMV RNA accumulation was linked to ABA signaling pathway. **a** Relative expression level of *Nb-L7/65* was up-regulated in the inoculated leaves of *Nb-CAR7*-overexpressed plants, but **b** down-regulated in both inoculated and upper leaves of *Nb-CAR7*-silenced plants. * represents $P < 0.05$ compared with control group

is a negative correlation between the expression of *Nb-CAR7* and accumulation of TMV RNA. As CAR proteins are involved in ABA signaling, our results showed that the effect of *Nb-CAR7* on TMV RNA accumulation was linked to the ABA signaling pathway. Further tests will be carried out to determine whether or not the down-regulation of *Nb-CAR7* is mediated by TMV-siRNA 22 nt (6285–6306).

The overexpression of *Nb-CAR7* through agrobacterium infiltration was restricted to plant cells in the infiltrated region. Therefore, TMV RNA accumulation was decreased only in the infiltrated leaves but not in the upper leaves after TMV was inoculated into the agrobacterium-infiltrated leaves. (Fig. 3c). RNA silencing of *Nb-CAR7* occurred in the upper leaves due to transient overexpression of *Nb-CAR7* in the infiltrated leaves, which led to a lower amount of *Nb-CAR7* in the upper leaves (Fig. 3d). Therefore, the accumulation of TMV RNA remained high in the upper leaves. In contrast, silencing of *Nb-CAR7* through TRV-based and VIGS-induced gene silencing occurred throughout the entire

plant. After TMV infection, silencing of *Nb-CAR7* led to a higher viral RNA accumulation in both inoculated and upper leaves of *Nb-CAR7*-silenced plants (Fig. 4b). Our results showed that the *Nb-CAR7* affects TMV replication. In future work, we will generate *Nb-CAR7*-transgenic plants to verify its effects on TMV infection.

ABA is a key plant hormone which has a great impact on plant defense against various pathogens (Alazem and Lin 2015; Sah et al. 2016; Alazem and Lin 2017). ABA enhances plant defense by shutting stomata and inducing callose deposition at cell walls (Ellinger et al. 2013). If a pathogen is successfully established inside a plant tissue, ABA induction can hinder plant defense by antagonizing other hormone pathways (Anderson et al. 2004; Yasuda et al. 2008). Therefore, ABA is labeled as a phase-specific modulator of defense responses. However, ABA induces different resistance mechanisms to viruses regardless of the induction time (Ton et al. 2009). ABA treatment decreases titers of *Bamboo mosaic virus* (BaMV) in inoculated leaves of *Arabidopsis* (Alazem et al. 2014). In addition, several mutants defective in



ABA signaling pathway exhibit enhanced accumulation of TMV crucifer strain in the upper systemically infected leaves of Arabidopsis but not in the inoculated leaves (Chen et al. 2013). There are a lot of reports about the increase of ABA content in virus-infected host plants, such as in CMV-infected *N. benthamiana*, BaMV-infected *A. thaliana* and *N. benthamiana* plants (Alazem et al. 2014), and in TMV-infected *N. tabacum* plants (Fraser and Whenham 1989). However, virus infection does not always induce an increase in ABA content in plants. For example, *Potato virus Y* does not induce an increase of ABA in resistant potato cultivar Sante but induced an increase of jasmonic acid (JA) within the first few hours after virus infection (Flis et al. 2005; Kovač et al. 2009). A resistant tomato cultivar that carries the *R*-gene *Tm-1* has a higher ABA content than a susceptible cultivar. However, the ABA content in the resistant tomato cultivar remains unchanged after TMV infection (Whenham et al. 1986; Baebler et al. 2014).

ABA has been linked to antiviral silencing pathway which interferes with virus accumulation (Alazem and Lin 2015; Alazem et al. 2017). It is also involved in the microRNA (miRNA) pathway and affects maturation and stability of miRNAs (Lu and Fedoroff 2000). Additionally, it induces callose deposition at plasmodesmata,

limiting viral cell-to-cell movement (Iglesias and Meins Jr 2000). ABA induces resistance against BaMV through Argonaute proteins (AGO 2 and 3), indicating that ABA regulates the expression of several members of the AGO family, and this regulation partially contributes to ABA-mediated resistance against viruses (Alazem et al. 2017). In this study, based on the high nucleotide sequence homology, the TMV-vsiRNA 22 nt (6285–6306) derived from the juxtaposition of UPD and TLS of TMV induced silencing of a host gene, *Nb-CAR7*, in the ABA signaling pathway. Downregulation of *Nb-CAR7* enhanced TMV RNA accumulation. In addition, the TMV-vsiRNA 22 nt (6285–6306) that targets *Nb-CAR7* for degradation possessed an adenosine at its 5' end. The AGO2 and AGO4 could be involved in this targeting as AGO2 and AGO4 preferentially recruit small RNAs with a 5' terminal adenosine (Mi et al. 2008).

Previous studies have shown that vsiRNA derived from a plant virus can target a host gene for regulation (Shi et al. 2016). Some siRNAs from satellite RNA of virus can also target host genes. For example, CMV Y-sat produces a 22 nt sequence that is complementary to tobacco magnesium protoporphyrin chelatase subunit I gene *ChlI* to disrupt chlorophyll biosynthesis, which results in

chlorotic symptom (Shimura et al. 2011). Not only targeting host genes, the vsiRNA has also been reported to target host long non-coding RNA to affect symptom development (Yang et al. 2019). The vsiRNAs generated from viroids could guide degradation of host mRNAs (Navarro et al. 2012). Also, a small RNA derived from the conserved region of *Potato spindle tuber viroid* (PSTVd) silences tomato *FRIGIDA-like* protein 3 gene (*FRL3*) and leads to early flowering of its host plants (Adkar-Purushothama et al. 2018). All these studies showed that vsiRNA could target host genes and affect disease symptoms development. In this study, the TMV-vsiRNA derived from the juxtaposition of UPD and TLS of TMV targets a host C2-domain ABA-related gene after virus infection. The results provide a new link of TMV-specific vsiRNAs regulating host genes during virus infection. The transcript level of *Nb-CAR7* was significantly decreased in TMV-infected plants, as compared with that of mock-inoculated and TMV-43A-inoculated plants, in which the 22 nt vsiRNAs are absent. Our results showed that the 22-nt TMV-vsiRNA regulates its target gene *Nb-CAR7* through mRNA degradation. Whether it also regulates this target gene through translation inhibition needs further study.

Conclusions

Our study discovered that TMV-vsiRNA could target a host gene *Nb-CAR7*. Overexpression of *Nb-CAR7* led to decreased TMV RNA accumulation in the inoculated leaves and silencing of *Nb-CAR7* enhanced TMV replication and resulted in a higher viral RNA accumulation. Moreover, the expression of *Nb-CAR7* was closely correlated with the expression levels of abscisic acid (ABA) responsive genes in plants, which indicated that the effect of this gene to TMV RNA accumulation in host plants may be mediated by ABA signaling pathway.

Methods

Plant inoculation and RNA isolation

Nicotiana benthamiana plants were grown in a plant growth room at 25 °C under a 16 h light/8 h dark photoperiod. Four-week-old plants were inoculated with 2 µg of in vitro transcribed RNAs of TMV and TMV-43A, respectively. Total RNA was isolated from infected plants using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The low molecular weight (LMW) RNA was isolated from infected leaves using polyethylene glycol (PEG) (An et al. 2013). After PEG precipitation, the supernatant was transferred to a new tube, and then 1/10 volume of NaOAC and two volumes of absolute ethanol were added. The mixture was incubated at -20 °C for 30 min and centrifuged at 12,000× g for 10 min at 4 °C. The pellet was washed twice with 70% ethanol, briefly air dried and dissolved in 20 µL of DEPC-treated water.

Northern blot hybridization

Low molecular weight (LMW) RNA samples were boiled for 5 min, then immediately chilled on ice for 5 min. For each RNA sample, an equal volume of formamide loading buffer (98% formamide, 40 mM EDTA, 0.05 mg/mL bromophenol blue, 0.05 mg/mL xylene cyanol FF) was added before loading. Treated samples were loaded onto denaturing 17% polyacrylamide gels and run at 200 mA. RNA was electro-blotted onto Hybond-N⁺ membrane (Amersham) in 0.5 × TBE buffer, immobilized onto the membrane by UV cross-linking, and hybridized overnight at 42 °C with DIG-labelled oligonucleotide probe corresponding to TMV (6285–6306 nt). The hybridized membranes were washed three times with 2 × SSC and 0.2% SDS for 20 min per wash at 42 °C and visualized by CSPD ready-to-use kit (Sigma-Aldrich). U6 RNA was used as an internal control.

Validation of *Nb-CAR7* cleavage by 5'-RLM-RACE

5'-RNA ligase-mediated rapid amplification of cDNA ends (5'-RLM-RACE) was performed to validate *Nb-CAR7* cleavage using the GeneRacer™ kit (Invitrogen) with the manufacturer's instructions modified as follows. Briefly, 100 ng total RNA was directly ligated to the RNA linker without prior treatment. After phenol/chloroform extraction and precipitation, first-strand cDNA was synthesized using Gene-Specific Primer (Additional file 1: Table S1). In first-round 5'-RACE reaction, 1 µL of primer was used including the GeneRacer 5' primer from the kit and synthesized Gene-Specific Primer (Additional file 1: Table S1) with cycling as described in the GeneRacer™ kit manual: 1 cycle of 94 °C for 2 min, then 5 cycles of 94 °C for 30 s and 72 °C for 1 min, then 5 cycles of 94 °C for 30 s and 70 °C for 1 min, then 20 cycles of 94 °C for 30 s and 68 °C for 1 min.

For the second-round 5'-RACE reaction, 1 µL of the first-round reaction and internal primers was used, which are GeneRacer™ Nested primer from the kit and the synthesized Gene-Specific-Nested primer (Additional file 1: Table S1) with cycling conditions mentioned above except for an extension time of 15 s. For this reaction, 5 µL of product was analyzed on agarose gel. The PCR amplified fragment of ~500 bp (Fig. 2d) was sequenced using an ABI3130XL sequencer with BigDye™ Terminator 3.1 Ready Reaction Cycling kit (Applied biosystems, USA) to confirm the cleavage of host *Nb-CAR7* gene.

Construction of *Nb-CAR7* over-expression and silencing vectors

The complete ORF of *Nb-CAR7* was inserted into pGreen vector with a GFP tag using primers *Nb-CAR7*-EcoRI-F and *Nb-CAR7*-BamHI-R to construct a *Nb-CAR7* over-expression vector termed as 35S-NbCAR7-GFP. The nucleotide sequence of *Nb-CAR7* (101–497 nt) was amplified

and ligated into pTRV2 vector with primers *Nb-CAR7*_(101–497)-EcoRI-F and *Nb-CAR7*_(101–497)-BamHI-R to construct a *Nb-CAR7* silencing vector. The primers sequences were listed in the Additional file 1: Table S1.

TRV-based expression system for gene silencing and agroinfiltration-based transient gene overexpression

Nb-CAR7 was silenced by TRV-based gene silencing system. For agroinfiltration, an equal volume of *Agrobacteria* containing pTRV1 or pTRV2:*Nb-CAR7* was mixed and infiltrated into two opposite leaves close to the bottom of four-week-old *N. benthamiana* plants using a 1-mL syringe. The agroinfiltration of pTRV1 with empty pTRV2 served as control group. In vitro transcribed TMV RNA (2 µg) was inoculated to the infiltrated leave of *Nb-CAR7*-silenced plants at 7 days post infiltration. Both inoculated and upper leaves were collected for detection of viral RNAs accumulation at 7 dpi.

For agroinfiltration-based transient gene overexpression of *Nb-CAR7*, *Agrobacteria* containing 35S-NbCAR7-GFP was infiltrated into two opposite leaves close to the bottom of four-week-old *N. benthamiana* plants using a 1-mL syringe. In vitro transcribed TMV RNA (2 µg) was inoculated to the infiltrated leave of plants at 3 days post infiltration. The inoculated leaves and upper leaves were collected at 7 dpi.

Agrobacterium infiltration

N. benthamiana plants were grown in plastic pots at 25 °C in a growth room under a 16 h light/8 h dark cycle. Related TRV-gene-silencing vectors and overexpression vectors were introduced into *Agrobacterium* strain GV3101 by electroporation (BIO-RAD, Hercules, CA, USA), respectively. A 5-mL culture was grown overnight at 28 °C in the appropriate antibiotic selection medium. The next day, the culture was inoculated into a 50 mL LB medium containing antibiotics, 10 mM MES and 20 µM acetosyringone. The culture was grown overnight in an incubator shaker at 28 °C. *Agrobacterium* cells were harvested and resuspended in infiltration media (10 mM MgCl₂, 10 mM MES, 200 µM acetosyringone), adjusted to an OD of 2.0 and left at room temperature for 3 h. *Agrobacterium* was infiltrated using a 1-mL syringe.

Quantitative RT-PCR (qRT-PCR) analysis

To detect the expression level of *Nb-CAR7* in plants, total RNA was isolated from mock-inoculated, TMV-infected, and TMV-43A-infected plants, respectively, at 4 dpi. cDNA was synthesized using Superscript III™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The complete open reading frame (ORF) of *Nb-CAR7* was amplified using KOD-plus-Neo DNA polymerase (Toyobo, Tokyo, Japan) with primers *Nb-CAR7*-F and *Nb-CAR7*-R.

For transient over-expression analysis, total RNA extracted from the 35S-NbCAR7-GFP-infiltrated leaves at 3 days post infiltration was used to determine the expression level of *Nb-CAR7*. qRT-PCR was performed in triplicates with primers *Nb-CAR7*-qF and *Nb-CAR7*-qR. *Actin* gene was used as an internal control with primers *Actin*-qF and *Actin*-qR.

For the TRV-induced gene silencing analysis, the ORF of *Nb-CAR7* was amplified from total RNA of infiltrated and upper leaves at 7 days post infiltration, respectively. RT-PCR was performed using Superscript III™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and KOD-plus-Neo DNA polymerase (Toyobo, Tokyo, Japan) with primers *Nb-CAR7*-F and *Nb-CAR7*-R.

To quantify TMV RNA accumulation in inoculated plants, total RNA isolated from inoculated leaves and upper leaves were extracted, respectively. Reverse transcription was performed using Superscript III Reverse Transcriptase kit (Life Technologies, Invitrogen) with primer TMV-R. qRT-PCR was performed in triplicates with primers TMV-qF and TMV-qR. To quantify the transcript level of *LTI65* in transiently *Nb-CAR7*-overexpressed leaves and *Nb-CAR7*-silenced plants, primers *Nb-LTI65*-F and *Nb-LTI65*-R were used to amplify the ORF of *Nb-LTI65*. qRT-PCR was performed in triplicates with primers *Nb-LTI65*-qF and *Nb-LTI65*-qR. *N. benthamiana actin* was used as an internal control.

All qRT-PCR analysis was performed using KAPA SYBR® FAST qPCR Master Mix (2X) Kit (KAPA Biosystem, Wilmington, MA, USA) on the CFX384 Real-Time PCR system (Bio-Rad). Each 5 µL-reaction mix contained 20 ng of cDNA, 200 nM of each pair of target primers and 2.5 µL of 2 × SYBR Green PCR Master Mix. PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. All primers sequences used for qRT-PCR reactions were listed in Additional file 1: Table S1.

Statistical analysis

All qRT-PCR expression assays were performed with three technical replicates from three independent biological replicates and repeated at least two times. The expression levels of *Nb-CAR7* and *Nb-LTI65*, and TMV viral RNAs accumulation level were normalized to that of *actin* gene, respectively. Data are expressed as mean ± standard error. The statistical data analysis was conducted via unpaired t-test. A difference was considered to be significant when $P < 0.05$ compared with control group.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s42483-020-00058-7>.

Additional file 1: Table S1. List of primer sequences used in this study.

Abbreviations

ABA: Absciscic acid; CAR: C2-domain absciscic acid-related; dpi: Days post inoculation; LTI65: Low-temperature-induced ABA responsive gene; PCR: Polymerase chain reaction; PK: Pseudoknot; RNA: Ribonucleic acid; RT: Reverse transcription; TLS: tRNA-like structure; TMV: Tobacco mosaic virus; UPD: Upstream pseudoknot domain; vsiRNA: Viral small interfering RNA

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Authors' contributions

SG performed experiments, analyzed the results and drafted the manuscript. SMW supervised the research and edited the manuscript. The authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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