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A tobamovirus RNA motif and TMV-Hel cooperatively suppress the host defense factor TRX

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Abstract

Viruses encode a limited number of proteins and often expropriate host factors for their successful infection and propagation. The molecular composition of the complex used for synthesis of the viral subgenomic (sg) RNAs remains largely unknown. In this study, an RNA motif (45 nt), equivalent to the sequence coding for the N-terminal portion of the tobamovirus coat protein (CP), known to be an enhancer-like element (tentatively designated as EL1) of the CP sg promoter, was used as a bait to capture bound proteins. *Nicotiana benthamiana* thioredoxin h-type 1 (NbTRXh1), a host defense factor with redox activity, was identified and its enzymatic active site was determined to be covered by EL1, thus blocking its reductase activity. In addition, the virus replicase Hel domain interacted with NbTRXh1, resulting in a decrease in NbTRXh1 protein accumulation in *N. benthamiana*. Thus, the viral RNA element and viral protein integrated their suppression of NbTRXh1 to facilitate viral CP sgRNA synthesis. Understanding this multiplexed regulatory mechanism between tobamovirus EL1 and associated proteins will assist in designing strategies for virus resistance and for optimization of the production of exogenous proteins under the control of the viral sg promoter.

Keywords Tobamovirus, Subgenomic promoter, Enhancer-like element, NbTRXh1, TMV-Hel

Background

Positive-strand (+) RNA viruses are the largest genetic category of eukaryotic viruses associated with severe diseases of plants, animals, and humans (Lindenbach et al. 2022). Tobacco mosaic virus (TMV) of the *Tobamovirus* genus (Adams et al. 2017) is a well-known pathogen with a wide host range and has been intensively studied as a model for plant virus research. TMV (U1 strain) harbors a monopartite single-stranded genomic RNA, comprising a minimum of four open reading frames (ORFs).

Two virus replication-related proteins (126/183 kDa) are translated from gRNA, while the movement protein (MP, 30 kDa) and coat protein (CP, 17.6 kDa) are translated from two subgenomic RNAs (sgRNAs). The MP and CP are involved in viral cell-to-cell movement and systemic infection, respectively (Hull 2014).

Virus replication is vital for infection of host cells. In TMV, the 126-kDa protein consists of a methyltransferase (Met) domain, an intervening region (IR), and a helicase domain (Hel) (Goelet et al. 1982; Ishibashi and Ishikawa 2016). Among these regions, TMV-Hel (located at 666–1116 amino acids) is crucial for viral replication (Goregaoker et al. 2001). TMV-Hel directly binds to double-stranded RNA as well as single-stranded RNA in an ATP-dependent manner, and assists with unwinding of the RNA duplexes (Goregaoker and Culver 2003). During virus multiplication, tobamovirus Hel exploits host factors to form viral

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replication complexes (VRCs) (Yamanaka et al. 2000; Nishikiori et al. 2011). In addition to its primary role in replication, TMV-Hel inhibits translation of viral gRNA by binding to the gRNA 5'-terminal region, leading to a switch from translation to replication (Kawamura-Nagaya et al. 2014). Moreover, TMV-Hel has RNA silencing suppressor activity (Wang et al. 2012). Wang et al. (2009) reported a function of TMV replicase in resisting the salicylic acid (SA)-mediated plant defense response. Considering the multiple functions of tobamovirus Hel, further elucidation of the molecular mechanisms of TMV-Hel may lead to an improved understanding of the viral life cycle (Wang et al. 2010).

During the life cycle of a (+) RNA virus, in addition to the viral proteins translated from gRNA, certain viral proteins, such as CPs and MPs, are generally translated from viral sgRNA. A previous study showed that different regulatory mechanisms existed in the control of gRNA replication and sgRNA synthesis (Li et al. 2007). The sgRNAs play critical roles in viral infection, fulfilling the regulatory functions in replication and transcription, and aiding in suppression of host immune responses (Shen and Miller 2004; Sztuba-Solinska et al. 2011; Pompon et al. 2017). Synthesis of sgRNAs has been extensively studied in plant systems, and different mechanisms for their generation have been proposed (Sztuba-Solińska et al. 2011). TMV sgRNA synthesis is achieved by internal initiation of transcription on the negative strand (Miller and Koev 2000).

The translational efficiency of sgRNAs is regulated by *cis*-regulatory elements within a specialized region in the genome, in which the subgenomic promoter (SGP) is a prominent factor. In plant viruses, SGP is generally located upstream or downstream of the transcription start site. The physical characterization of SGP is crucial for heterologous protein expression via a virus-based vector. In general, SGP comprises a proximal 'core' promoter and a distal 'full' promoter (Chattopadhyay et al. 2020). TMV-MP and -CP SGPs have been mapped, and the minimal CP-SGP is located between nucleotides (−)69 and (+)12, whereas the boundaries of the fully active promoter are located between nucleotides (−)157 and (+)54 (Grdzelišvili et al. 2000). In addition, a potential enhancer element (located at nucleotides +25 to +55) has been identified (Shivprasad et al. 1999; Man and Epel 2004). Furthermore, the secondary structure of SGP, such as a stem-loop structure, contributes to the promoter activity in TMV, turnip crinkle virus, red clover necrotic mosaic virus, and barley yellow dwarf virus (Zavriev et al. 1996; Koev et al. 1999; Wang et al. 1999; Grdzelišvili et al. 2000; Koev and Miller 2000), indicating the complex regulatory mechanism for efficient virus sgRNA synthesis.

During virus infection, the virus recruits host factors such as eukaryotic translation initiation factor 4E (Truniger et al. 2017; Ojha et al. 2024), glyceraldehyde 3-phosphate dehydrogenase protein (Wang and Nagy 2008), and photosystem II oxygen-evolving complex protein (Huang et al. 2021), functioning in viral replication. Although some host factors that bind to gRNA regulatory elements or the negative strand of viral RNA have been identified, the mechanism is not clear and, to the best of our knowledge, limited research has focused on the functional binding of host factors to sgRNA-related *cis*-regulatory elements. Therefore, understanding the mechanisms and components involved in the initiation and regulation of sgRNA synthesis will be beneficial for the control of viruses and optimization of viral infectious clone vectors.

The thioredoxin (TRX) antioxidant system contains multifunctional small redox proteins that are mainly involved in regulation of the redox environment in living cells (Mata-Perez and Spoel 2019). TRXh family consists of subgroups that vary in biochemical properties, subcellular localization, and reduction pathways, indicating the diversity of their functions in plants (Gelhay et al. 2004; Kumari et al. 2021). Several TRX-encoding genes from different plant species, such as *AtTrxh5*, *ZmTrxh*, *CaTrxh1*, *OsTRXh1*, *TaTrxh1*, and *GbNRX1*, play regulatory roles in diverse processes that are mainly involved in defense responses and pathogen interactions (Zhang et al. 2011; Kneeshaw et al. 2014; Li et al. 2016; Luna-Riviero et al. 2016; Liu et al. 2017; Shi et al. 2021). *AtTrxh5* enhances SA-induced immune gene expression by modulating the conformational state of NPR1 (Tada et al. 2008; Kneeshaw et al. 2014). In tobacco, *NtTRXh3*, a TRXh-like protein, is localized exclusively to chloroplasts. *NtTRXh3* has redox activity and reduces the pathogenicity of TMV and CMV, and protects cells from oxidative damage (Sun et al. 2010). The protein *NbTRXh1*, which shows 30.92% identity in amino acid sequence with that of *NtTRXh3*, has been reported to be induced by TMV infection (Das et al. 2019). Jiang et al. (2022) observed that the barley stripe mosaic virus ν b protein interacts with *NbTRXh1* to weaken its reductase activity, thereby suppressing SA-mediated plant resistance to assist viral cell-to-cell movement, indicating that *NbTRXh1* is also a target for virus infection.

Based on the vital role of the virus SGP in sgRNA accumulation, together with its crucial function in regulating exogenous gene expression in the virus vector, an enhancer-like element (45 nt) of tobamovirus CP-SGP (designated EL1) was screened in a preliminary mining experiment with the TMV 30B-derived vector in *N. benthamiana* (Additional file 1: Figure S1a; Additional file 2: Table S1). The bell pepper mottle virus EL1 strain (BPMV-EL1) showed potential application in

optimization of the TMV genome-based vector (Additional file 1: Figure S1b–d). In this study, we systematically studied the molecular mechanism of the BPMV-EL1 RNA motif underlying its high activity in viral expression. A host defense-related factor, NbTRXh1, was isolated and the EL1 RNA motif specifically bound to the catalytic center of NbTRXh1 to inhibit its reductase activity. In addition, the TMV-Hel domain interacted with NbTRXh1 and resulted in decreased accumulation of NbTRXh1. The present investigation of the interactions among the virus RNA motif, TMV-Hel, and host factor NbTRXh1 contributes to an improved understanding of the complex regulatory strategies between viruses and plant hosts.

Results

BPMV-EL1 motif physically interacts with the host protein NbTRXh1

The virus SGP is considered to promote gene expression by interacting with proteins such as replicase-associated

proteins, and the host factors cannot be excluded. Therefore, to identify the potential plant factors associated with the function of the EL1 motif in controlling gene expression, and based on the report by Ramanathan et al. (2018) using the RNA–protein interaction detection (RaPID) method in HEK293T living cells, we developed a modified RaPID technique used in a plant agroinfiltration transient system (designated RaPIDp) (Fig. 1a). In this method, the stable RNA component produced from the green fluorescent protein (GFP) gene 3' untranslated region (UTR) was confirmed by reverse transcription–PCR with the primer pair R-motif-S/R-motif-R (Additional file 2: Table S2). Next, a 45-nt fragment of the BPMV-EL1 RNA sequence was used as a bait and several potential BPMV-EL1-binding proteins were generated (Additional file 2: Table S3). Among these proteins, *N. benthamiana* thioredoxin h-type 1 (NbTRXh1; GenBank: ACV52592.1) with a low molecular weight was selected and expressed in *E. coli* for detection of the interaction in vitro. An RNA electrophoretic mobility shift assay

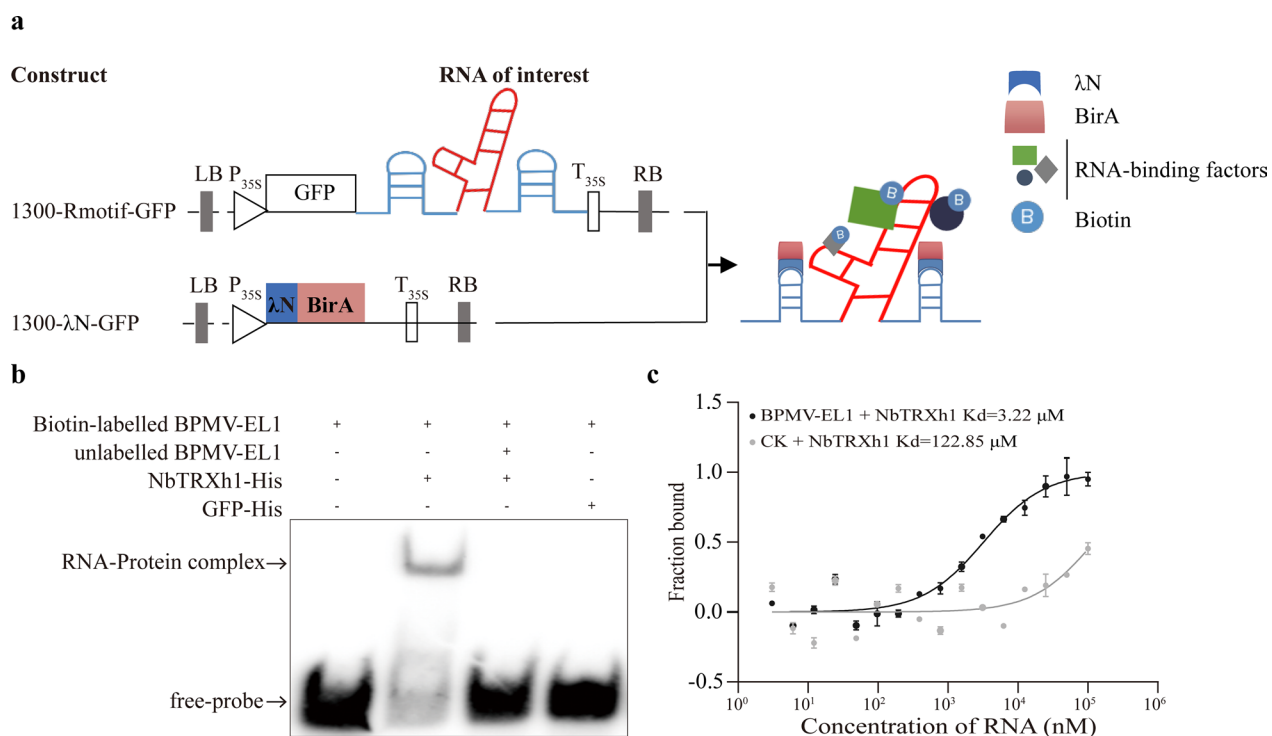


Fig. 1 Mining of potential factors bound to tobamovirus EL1 RNA motif by RaPIDp method. **a** Schematic showing of constructs used in mining plant proteins interacting with the target RNA sequence via agroinfiltration transient expression system in *N. benthamiana*. In plant binary construct 1300-Rmotif-GFP, the interested RNA fragment was flanked by the BoxB RNA motifs, and located at downstream of GFP-ORF and transcribed as a 3' UTR of GFP. 1300-λN-BirA vector was used for expression of λN-BirA fusion biotin ligase driven by P_{35S} promoter. Due to the specific binding of BoxB with λN peptide, the potential proteins associated with interested RNA fragment were also biotinylated, thus it is feasible to further streptavidin capture following mass spectrometry. **b, c** In vitro detecting the interaction of BPMV-EL1 motif with NbTRXh1. RNA-EMSA assay of NbTRXh1-binding activity to BPMV-EL1 motif. The binding signal was only displayed in the treatment of 1 μg NbTRXh1-His fusion protein with the biotin-labelled BPMV-EL1 probe. The biotin-unlabelled RNA probe was used as a competitive cold probe, and GFP as a negative protein control (**b**). The MST result showed that NbTRXh1 was directly bound to BPMV-EL1 with a high affinity (K_d=3.22 μM). The 45 nt fragment of unrelative RNA (CK) was used as a negative control (**c**). The EMSA and MST were performed with three biological repetitions, and the results were consistent

(EMSA) showed the physical interaction of NbTRXh1 with the BPMV-EL1 RNA motif (Fig. 1b). Furthermore, a microscale thermophoresis (MST) assay revealed strong binding activity between NbTRXh1 and BPMV-EL1 (Fig. 1c).

To further confirm the interaction of BPMV-EL1 with NbTRXh1, utilizing the principle of a bimolecular fluorescence complementation assay (BiFC), we devised a novel method (designated BiFCrp) to test the in vivo interaction of the target RNA with potential

proteins (Fig. 2a). In the BiFCrp method, the previously reported specific binding characteristics of the BoxB RNA motif with the λ N peptide were utilized (Ramanathan et al. 2018), in which the target RNA sequence was flanked by the BoxB RNA motif. To evaluate the BiFCrp method, the previously reported binding of UPD (a conserved upstream pseudoknot domain of the TMV 3' UTR) with EF1A (Eukaryotic Elongation Factor 1 A) (Zeenko et al. 2002) served as a positive control (Additional file 1: Figure S2), followed by detection of

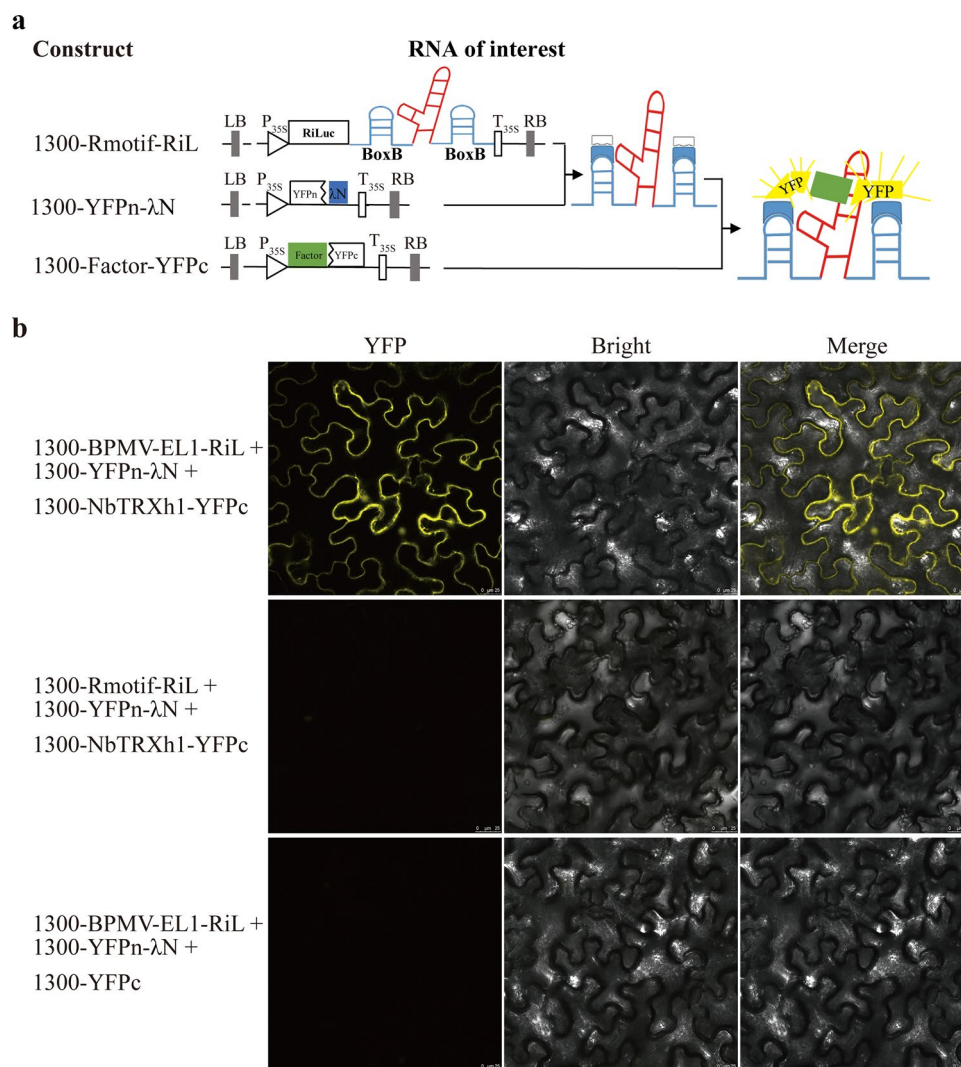


Fig. 2 Detecting the interaction of BPMV-EL1 with NbTRXh1 by in vivo BiFCrp method. **a** The schematic flow of the modified BiFC for in vivo monitoring an interaction of target RNA sequence with potential proteins (BiFCrp). The interested RNA sequence flanked by BoxB motif was transcribed as 3' UTR of RiLuc reporter in 1300-Rmotif-RiL construct, and YFPn- λ N fusion protein was expressed from 1300-YFPn- λ N. Correspondingly, the candidate factor fused to YFPc in 1300-Factor-YFPc construct. Benefiting from the combination of BoxB with λ N motif, in the leaf sample co-infiltrated with the *Agrobacterium* combination mix of 1300-Rmotif-RiL, 1300-YFPn- λ N, and 1300-Factor-YFPc three constructs, the interested RNA which linked with BoxB motif will indirectly fused with YFPn. Thus, whether the sample emits yellow fluorescence depends on the binding of target RNA to the host factor. **b** Using BiFCrp method to test the interaction between BPMV-EL1 and NbTRXh1 in *N. benthamiana* leaf cells. In the co-infiltration mix that contains 1300-Rmotif-RiL lacking target RNA sequence, or 1300-YFPc empty vector was used as negative controls, respectively. Scale bars: 25 μ m. The BiFCrp was performed with three biological and technical replicates, and the results were consistent

the interaction of BPMV-EL1 with NbTRXh1. The BiFC in vivo analysis revealed that the yellow fluorescent protein (YFP) signal was observed in *N. benthamiana* leaves co-infiltrated with *Agrobacterium* harboring the constructs 1300-BPMV-EL1-RiL, 1300-YFPn- λ N, and 1300-NbTRXh1-YFPc, but not in the negative control treatments comprising the 1300-Rmotif-RiL construct lacking the target RNA, or the 1300-YFPc vector (Fig. 2b). The transcription level of EL1 and the protein expression levels of the various constructs were measured (Additional file 1: Figure S3a, b). To test the difference between the YFPn- and YFPc-fused constructs, a further set of 1300-BPMV-EL1-RiL, 1300- λ N-YFPc, and 1300-YFPn-NbTRXh1 constructs were generated, in which the position of YFPn or YFPc in the corresponding vectors was transposed, but similar results were obtained (Additional file 1: Figure S4). These data demonstrated the physical binding activity of the BPMV-EL1 RNA motif to NbTRXh1.

Altering expression of *NbTRXh1* in *N. benthamiana* significantly influences virus replication and GFP expression

Based on the direct interaction between the virus EL1 motif and the host factor NbTRXh1, we further investigated the impact of NbTRXh1 on viral expression of foreign genes produced from a TMV genome-derived vector. Its hairpin gene-silencing vector (*NbTRXh1*-RNAi) and cauliflower mosaic virus (CaMV) 35S-driven overexpression construct (*NbTRXh1*-OE) were generated. In the *Agrobacterium*-mediated transient expression system in *N. benthamiana* leaves, pre-inoculation of agrobacterial cells containing the *NbTRXh1*-RNAi or *NbTRXh1*-OE plasmid for 2 days was performed, and the change in *NbTRXh1* transcript levels was confirmed (Fig. 3a, e), followed by injection of *Agrobacterium* harboring the pAT-BPMV-EL1-GFP or pAT-BPMV-EL1-CP plasmid. After 2–3 days, GFP or CP expression was separately detected. Promotion of GFP and CP expression

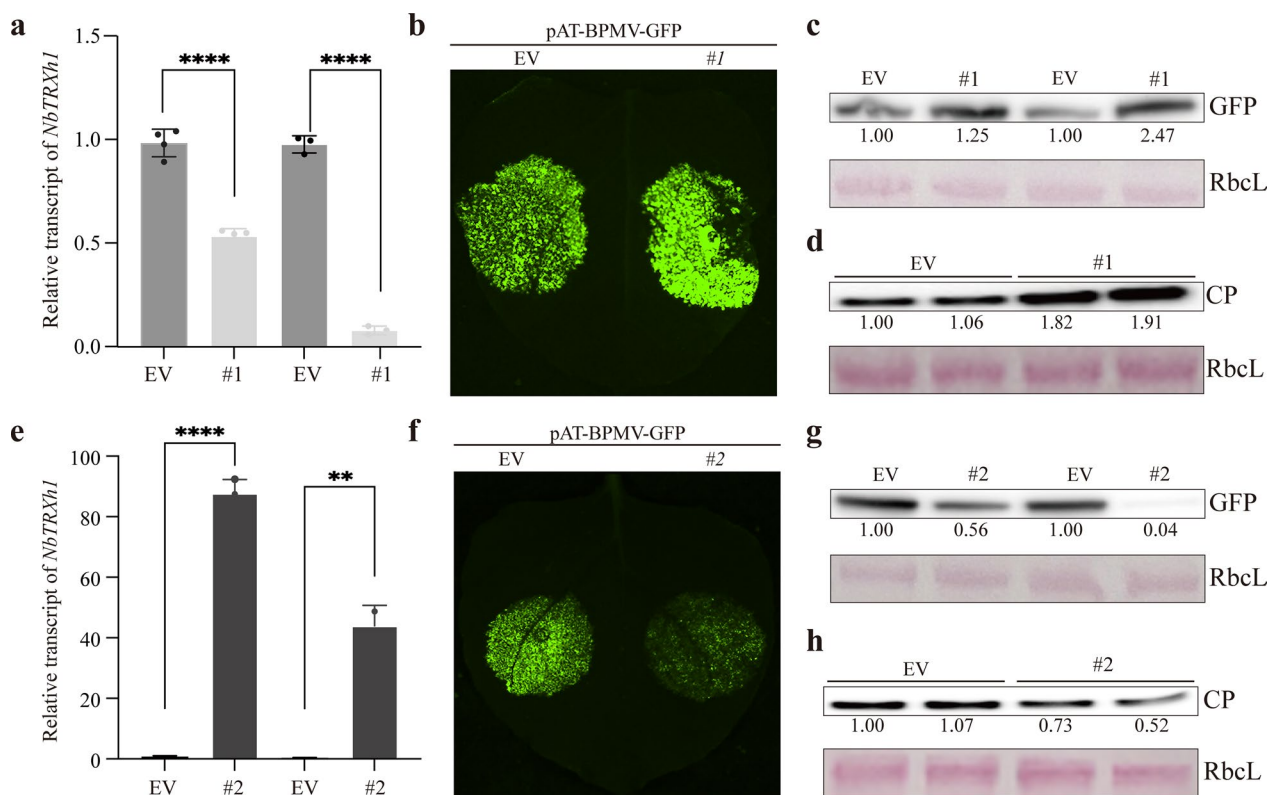


Fig. 3 The impact of transient *NbTRXh1*-RNAi (#1) or -overexpressing (#2) on the viral expression of GFP/CP. **a–d** Pre-infiltrated with *NbTRXh1*-RNAi (#1) containing *Agrobacterium* accelerated the product of GFP/CP expressed from pAT-BPMV-EL1-GFP or -CP construct in *N. benthamiana*. **e–h** Prior overexpression of *NbTRXh1* (#2) inhibited viral GFP- or CP-expression. **a** and **e** The changed transcription of *NbTRXh1* in prior samples by RT-qPCR at 2 days post inoculation (dpi). Data were the mean value of three independent experiments from 9 leaf pool samples, and analyzed by Student's *t* test (** $P < 0.01$; **** $P < 0.0001$). *ACTIN* gene was used as an internal control. Comparison of GFP fluorescence signals with half-leaf method at 2 dpi (**b** and **f**), and western blot analyses the accumulation of GFP (**c** and **g**) or CP (**d** and **h**). In the whole leaf agroinfiltrated samples, the quantity of CP at 3 dpi represents virus replication. The antiserum against GFP or TMV-CP was used, and RbcL was a loading control. EV: the empty vectors of pCambia-HAN and pCambia-1300 were used as the negative control of #1 and #2, respectively. Above experiments were performed three biological replicates with similar results

was observed in the leaves pretreated with *NbTRXh1*-RNAi (Fig. 3b–d). By contrast, in *NbTRXh1*-OE-pretreated leaves, the increase in *NbTRXh1* expression level distinctly inhibited the accumulation of GFP or CP (Fig. 3f–h). In the pAT-BPMV-EL1-CP-inoculated leaves, various viral infection symptoms developed (Additional file 1: Figure S5), which was consistent with the change in TMV-CP accumulation. Therefore, these results showed that *NbTRXh1* negatively regulated virus replication and TMV-based viral expression, and demonstrated that *NbTRXh1* is an important factor mediating the antiviral response in *N. benthamiana* leaves.

Dissection of the key region of BPMV-EL1 for binding to *NbTRXh1*

The interaction between the virus BPMV-EL1 and the host antiviral factor *NbTRXh1* motivated in-depth research on the mechanism. Given that RNA promoters generally contain the regions recognized by viral RNA polymerase or associated subunits, we identified the crucial region of BPMV-EL1 by separately mutating the nucleic acids from nt 1–15 (BPMV-EL1a), nt 16–30 (BPMV-EL1b), and nt 31–45 (BPMV-EL1c) (Fig. 4a). The EMSA binding activity revealed that, compared with the binding signal of the wild-type BPMV-EL1 with *NbTRXh1*, no signal was shown by the BPMV-EL1b mutant, indicating that the interaction with *NbTRXh1*

was disrupted, whereas the BPMV-EL1a and BPMV-EL1c mutants retained binding activity with observable signals (Fig. 4b). Consistent with this finding, the weakest interaction signal in MST assays was between BPMV-EL1b and *NbTRXh1* (Fig. 4c). These results showed that nt 16–30 of BPMV-EL1 included the specific binding site with *NbTRXh1*. This finding encouraged us to test the role of BPMV-EL1b in viral expression of the GFP foreign gene. Compared with the GFP fluorescence signal produced from the pAT-BPMV-EL1-GFP construct containing the wild-type BPMV-EL1, no GFP signal was detected from the pAT-BPMV-EL1b-GFP construct containing BPMV-EL1b (Fig. 4d). This result was further confirmed by RT-qPCR and a western blot assay (Fig. 4e–f). Therefore, these data showed that the internal sequences (15 nt) of BPMV-EL1 were responsible for binding to *NbTRXh1*, and that the binding site was essential for viral expression of GFP.

The internal sequence (nt 16–30) of BPMV-EL1 binds to the reducing activity center ⁴²WCGPC⁴⁶ of *NbTRXh1*, resulting in loss of its reductase activity

The physical binding activity of BPMV-EL1 to *NbTRXh1* inspired us to examine if this interaction plays a crucial role in the response to biological interactions. As previously reported, RT-qPCR results showed that the transcription of *NbTRXh1* was induced at 24–72 h after

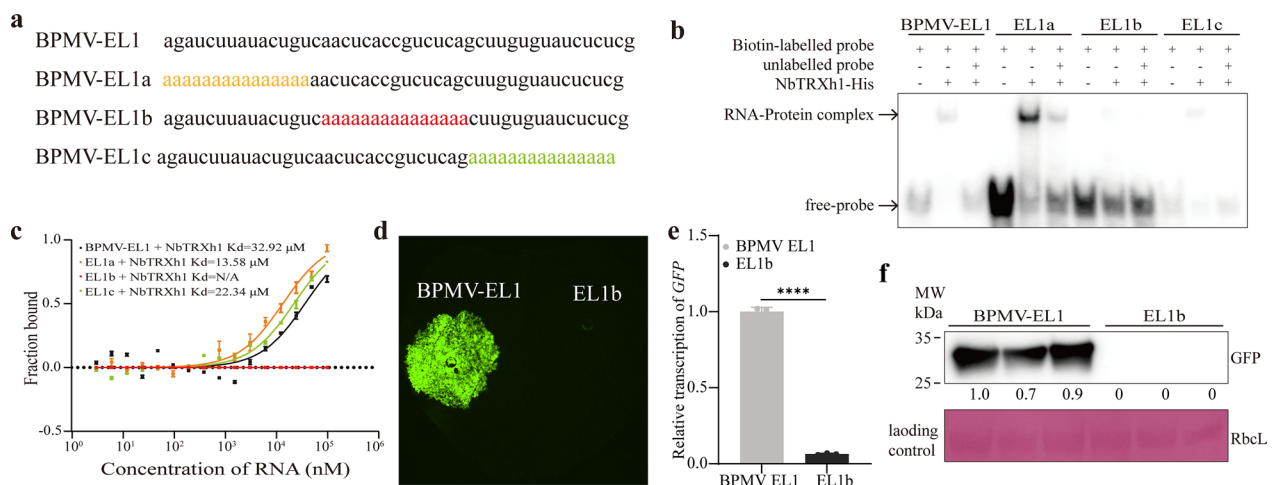


Fig. 4 Identification of the crucial region of BPMV-EL1 specifically binding to *NbTRXh1*. **a** The sequences of BPMV-EL1 and mutants. The mutated region at 1–15, 16–30, and 31–45 nt was corresponding to the mutants of BPMV-EL1a, BPMV-EL1b, and BPMV-EL1c, respectively. **b** Comparison of the binding activity of 1 μg *NbTRXh1*-His fusion protein to BPMV-EL1 motif wild type probe or its mutants in EMSA. Among the mutant probes (EL1a, EL1b, and EL1c), only EL1b completely abolished the RNA–protein interaction. The biotin-unlabeled probes were used as competitive cold probe. **c** MST measurement of the interaction between *NbTRXh1* and BPMV-EL1 or BPMV-EL1a–c mutants. **d–f** The influence of EL1b mutant in TMV-based transient viral GFP expression. **d** Comparing the green fluorescence of GFP signal. **e** RT-qPCR detecting the transcription levels of GFP produced from pAT-BPMV-EL1-GFP and pAT-BPMV-EL1b-GFP construct. The data are mean values (n = 3), analyzed by GraphPad Prism 9 Student's *t* test (**** *P* < 0.0001). **f** protein accumulation by western blotting immunoassay via half-leaf method in *N. benthamiana*. Each sample was collected from 9 leaves, and the immune signals from western blot were quantitatively analyzed with ImageJ. Above experiments of EMSA, MST, and GFP expression were performed independently at least three biological repeats, and showed similar results

abrasion inoculation with TMV in *N. benthamiana* leaves (Additional file 1: Figure S6a, b). The NbTRXh1 protein was mainly localized to the cell membrane and nucleus (Additional file 1: Figure S6c–e), which differed from the chloroplast location of NtTRXh3, indicating the diversity of their roles. To test if the RNA–protein interaction can affect the reductase activity of NbTRXh1, the activity of the expressed NbTRXh1-His fusion protein purified from *E. coli* was first determined by an in vitro insulin reduction assay (Holmgren 1979). Compared with the negative control (PBS buffer), which showed no substantial increase in absorbance over time, the recombinant NbTRXh1-His fusion protein showed significant redox activity as indicated by the substantial increase in absorbance within 80 min (Fig. 5a). As two reactive cysteine residues are present in the conserved motif of thioredoxins, a mutant construct NbTRXh1_{ss} (⁴²WCGPC⁴⁶ mutated into ⁴²WSGPS⁴⁶) was generated. The high reductase activity shown by wild-type NbTRXh1 was completely eliminated in the NbTRXh1_{ss}-His mutant (Fig. 5b), demonstrating that the ⁴²WCGPC⁴⁶ motif of NbTRXh1 is necessary for its reductase activity.

Interestingly, compared with the reductive activity of wild-type NbTRXh1 in the insulin reduction assay, this activity was inactivated when NbTRXh1 was pre-incubated with BPMV-EL1 for 30 min, showing a significantly suppressed catalytic activity; however, pretreatment of NbTRXh1 with the BPMV-EL1b mutant motif had no impact on the activity of NbTRXh1 (Fig. 5b; Additional file 1: Figure S7a). Therefore, it was speculated that the critical binding site of BPMV-EL1 to NbTRXh1 may be located in its catalytic center (⁴²WCGPC⁴⁶). Thus, we detected the binding activity of BPMV-EL1 to the NbTRXh1_{ss} mutant. As expected, the MST assay showed that, compared with the high affinity of wild-type NbTRXh1 for BPMV-EL1, no binding signal was detected in the reaction with the NbTRXh1_{ss} mutant, and the EMSA results similarly

revealed a loss of binding activity of the NbTRXh1_{ss} mutant to the BPMV-EL1 motif (Fig. 5c, d). In addition, the results of an in vivo BiFCr experiment confirmed that the binding site of BPMV-EL1 to NbTRXh1 was located in the conserved motif ⁴²WCGPC⁴⁶ of NbTRXh1 (Fig. 5e; Additional file 1: Figure S3c, d, S7b). Collectively, these results demonstrated that the catalytic center (⁴²WCGPC⁴⁶) of NbTRXh1 is the target of BPMV-EL1, and the binding of NbTRXh1 to BPMV-EL1 significantly suppresses its reductase activity.

TMV-Hel participates in the mutual regulation between BPMV-EL1 and NbTRXh1 motif

The functional regulation between the viral EL1 motif and the host NbTRXh1 factor prompted us to speculate whether the interaction is also associated with viral proteins. Given that TMV-Hel is often closely associated with the virus sequence, such as by binding to the 5' UTR gRNA sequence, to regulate virus replication (Kawamura-Nagaya et al. 2014), the interaction between TMV-Hel and EL1 was assessed. The results of a BiFCr experiment (Fig. 6a; Additional file 1: Figure S3e, f, S8a), together with MST and EMSA data (Fig. 6b; Additional file 1: Figure S8c, d), revealed the binding activity between BPMV-EL1 and TMV-Hel, indicating their physical interaction.

The involvement of TMV-Hel in this process was first detected by performing a BiFC assay. The results verified physical binding between TMV-Hel and NbTRXh1 (Fig. 6c; Additional file 1: Figure S3g, S8b), and their interaction was further confirmed by a GST pull-down assay (Fig. 6d). Furthermore, the subcellular co-localization of NbTRXh1 and TMV-Hel was observed in the cell membrane and nucleus (Fig. 6e–f). These results suggested that TMV-Hel, which is regularly involved in virus replication, also has a function associated with the host defense factor NbTRXh1.

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Fig. 5 The effect of BPMV-EL1 bound to NbTRXh1 on the reductase activity of NbTRXh1. **a** The reduction activity of NbTRXh1-His fusion protein detected by insulin reduction assay. **b** Comparative analysis of the reduction activity under different treatments. Compared with the high reduction activity of 5 μ M NbTRXh1-His, the treatments with NbTRXh1_{ss} mutant (⁴²WSGPS⁴⁶) or adding of 10 nM BPMV-EL1 in the reaction mix showed almost no or significantly reduced reduction activity, respectively. The experiments were performed independently for three times and showed similar results. **c** MST measurement of the interaction between NbTRXh1 wild type or NbTRXh1_{ss} mutant with BPMV-EL1 motif. This experiment was performed independently for twice and showed similar results. **d** Binding activity of NbTRXh1 or NbTRXh1_{ss} to BPMV-EL1 by EMSA. The gradient of NbTRXh1-His (1, 2, and 4 μ g) specifically bound to the biotinylated BPMV-EL1 probe (10 nM), resulted in the retardant band with a gradient signal, while the signal was disappeared in mutated NbTRXh1_{ss}-His (2, 4 μ g) contained samples. No signal was detected in the unlabeled cold probe of BPMV-EL1 (500 nM) competitive sample. **e** By BiFCr in vivo analysis, the fluorescence signal representing the binding activity of BPMV-EL1 to NbTRXh1 was quenched in the NbTRXh1_{ss} alternative co-infiltration sample. The mix of co-infiltration containing 1300-YFPc or 1300-Rmotif-RIL lacking BPMV-EL1 was used as negative control. Scale bars: 25 μ m. This experiment was performed independently for three times and showed similar results

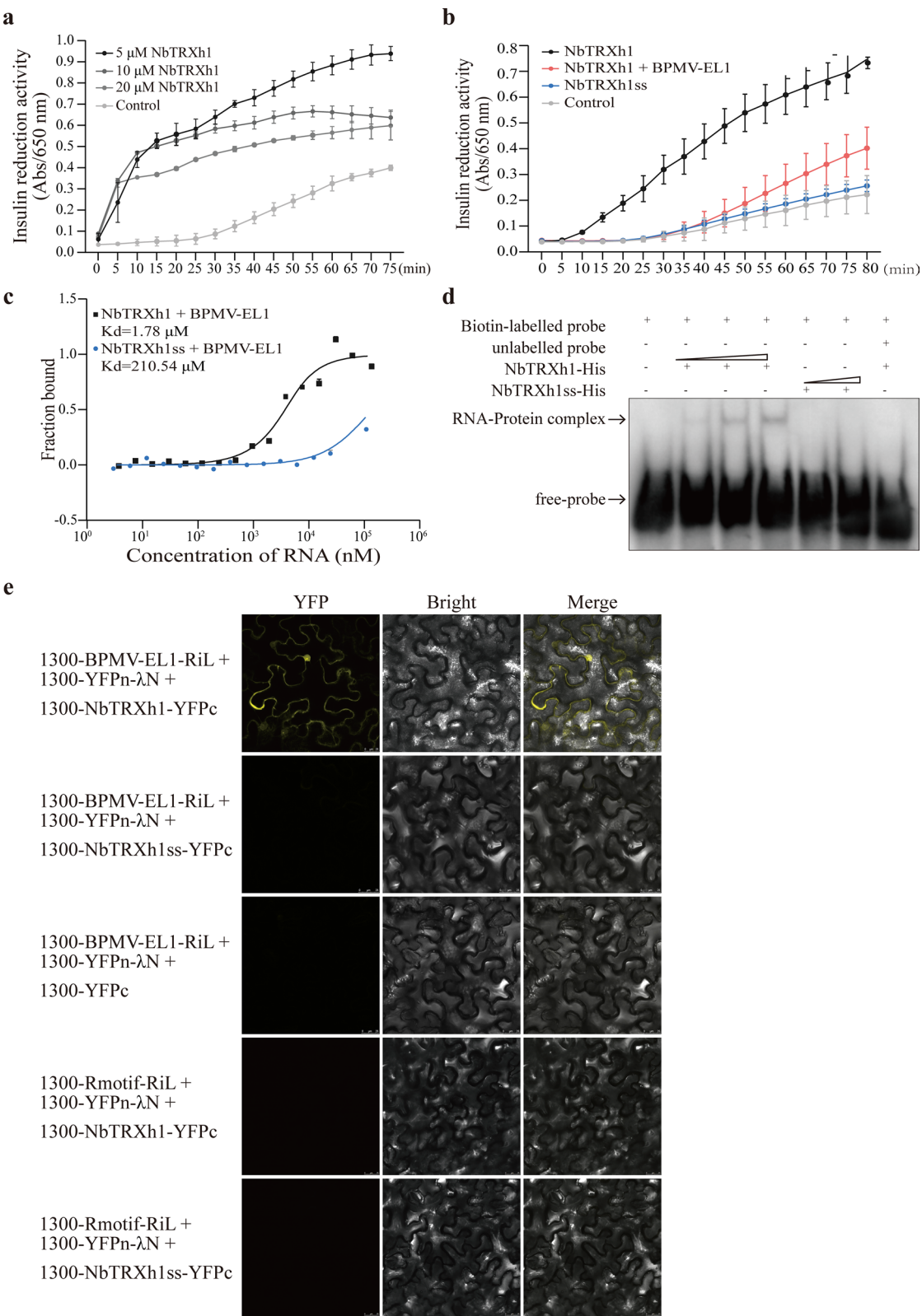


Fig. 5 (See legend on previous page.)

TMV-Hel suppresses accumulation of NbTRXh1 protein via their physical interaction in *N. benthamiana*

To determine the role of TMV-Hel in the tripartite interactions, the catalytic activity of NbTRXh1 was first examined. No significant effect of TMV-Hel on NbTRXh1 activity was detected (Fig. 7a). It was speculated that a competitive relationship between TMV-Hel and NbTRXh1 may influence their interaction with the BPMV-EL1 motif. The BPMV-EL1 motif and its EL1a–c mutants were used to identify the binding site of TMV-Hel with BPMV-EL1; the MST results showed that BPMV-EL1c was the critical region for the binding of TMV-Hel to BPMV-EL1 (Fig. 7b). Thus, it differed from the NbTRXh1-binding site located in BPMV-EL1b, indicating that no competition between TMV-Hel and NbTRXh1 influences their binding to the BPMV-EL1 motif.

Our previous experimental results for the TMV-induced expression pattern of NbTRXh1 were only detected at the transcription level. However, no signal was detectable, and therefore it was difficult to detect changes in the NbTRXh1 protein status, using the NbTRXh1 antibody. We wondered if the accumulation of NbTRXh1 was affected by TMV-Hel. Accordingly, the expression of the NbTRXh1 protein was tested again. Using a half-leaf method, *N. benthamiana* was co-infiltrated with *Agrobacterium* harboring the p1300-NbTRXh1-His construct and that harboring p1300-TMV-Hel-Flag or the p1300-GFP-His control plasmid, and the accumulation of the NbTRXh1-His fusion protein was determined by western blotting with a tagged antibody. As might be expected, the amount of NbTRXh1-His protein was significantly reduced by addition of the p1300-TMV-Hel-Flag plasmid compared with that observed under co-infiltration with the control p1300-GFP-His (Fig. 7c). Furthermore, with the addition of the p1300-TMV-Hel-Flag plasmid, reduced accumulation of the NbTRXh1-GFP fusion protein was observed by comparing the GFP fluorescence signal and immunoassay separately (Fig. 7d, e). To test if this function of

TMV-Hel is a general characteristic, p1300-GFP-His instead of p1300-NbTRXh1-His was used in the co-infiltration experiment, and no change in GFP expression was detected with or without 1300-TMV-Hel-His (Additional file 1: Figure S9a, b), suggesting that TMV-Hel specifically inhibited NbTRXh1 accumulation. Conversely, no significant effect of NbTRXh1 on TMV-Hel expression was observed (Additional file 1: Figure S10). Collectively, these results demonstrated that, in the tripartite interactions, TMV-Hel binds to the host defense factor NbTRXh1 and plays a role in specifically suppressing NbTRXh1 accumulation.

Discussion

Survival of RNA viruses, which have a minuscule genome, in plant cells requires multiple expression strategies and regulatory mechanisms to utilize host resources, and to confront and neutralize host defenses (Li and Nagy 2011; Nagy and Pogany 2011). The expression of multiple viral proteins, including CP, MP, and RNA-silencing suppressor-associated factors, driven by the virus SGP play important roles in viral replication and infectivity (Newburn and White 2015). However, their specific regulatory mechanisms remain obscure, especially the SGP-related RNA motif that directly binds to virus/host factors, and require further study to elucidate the responsible mechanisms. Differing from a DNA promoter, the RNA promoter of the viral SGP usually has the characteristics of a short sequence, complex secondary structure, and sometimes drives more than one gene (Koev and Miller 2000), reflecting a strict and complex regulatory mechanism. Thus, identification of efficient SGP-related *cis*-regulatory elements and investigation of their mechanisms are beneficial for virus-resistance engineering and improving plant-based products via viral expression.

In this study, based on a TMV genome-derived vector, we identified an enhancer-like element RNA motif (BPMV-EL1) from 33 species in the genus *Tobamovirus*, and further investigated its regulatory mechanism

(See figure on next page.)

Fig. 6 Detecting the physical interaction of TMV-Hel with both BPMV-EL1 motif and NbTRXh1. **a, b** The binding activity of TMV-Hel with BPMV-EL1 motif determined by BiFCr and MST assay, respectively. In BiFCr co-infiltration treatments, the 1300-Rmotif-RiL empty vector lacking the target RNA sequence and 1300-YFPc empty vector were used as negative controls. Scale bars: 50 μ m. The MST result showed that BPMV-EL1 was directly bound to TMV-Hel with a high affinity ($k_d = 17.9$ nM). BSA with BPMV-EL1 ($k_d = N/A$) was used as a negative control, while NbTRXh1 with BPMV-EL1 ($k_d = 5.32$ nM) was used as a positive control. Above experiments were performed independently for three times and showed similar results. **c** The interaction between TMV-Hel and NbTRXh1 factor was analyzed by BiFC assay in *N. benthamiana* leaf cells. Compared with the strong binding signal displayed between TMV-Hel with NbTRXh1 wild type, a relative weak signal was shown from NbTRXh1ss mutant. The reconstructed YFP signal was visualized by confocal microscope at 2 dpi. Empty vector of 1300-YFPc was used as a negative control. Scale bars: 50 μ m. **d** GST pull-down assay. **e–f** Subcellular co-localization analysis of NbTRXh1-BFP and TMV-Hel-GFP fusion proteins in *N. benthamiana* cells. **e** Membrane co-localization of NbTRXh1 and TMV Hel. **f** Nuclear co-localization. PIP2A (red, membrane marker) and H2B (red, nuclear marker) were used as markers for plasma membrane and nucleus, respectively. Scale bars: 25 μ m. These experiments were performed independently three times and showed similar results

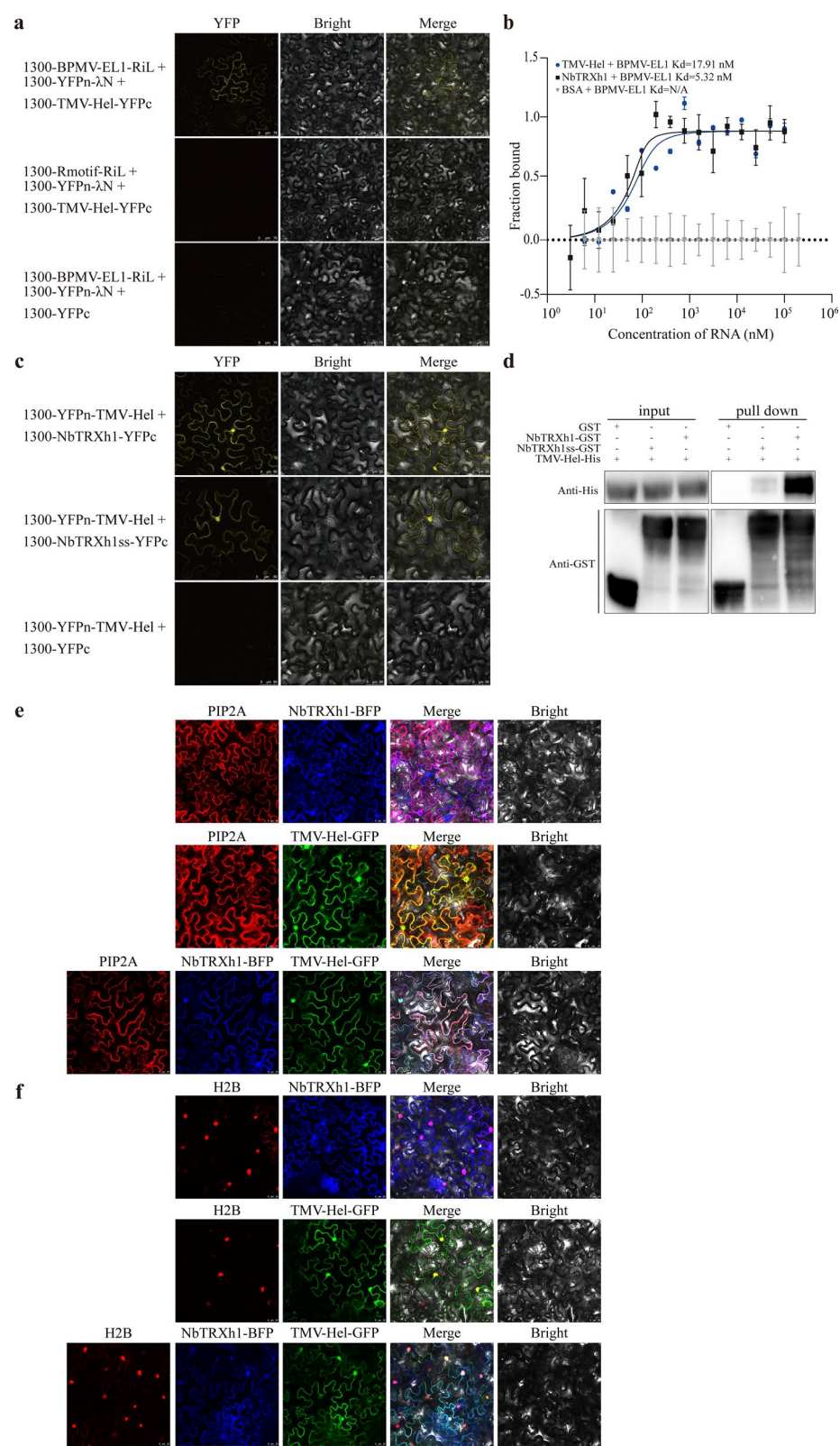


Fig. 6 (See legend on previous page.)

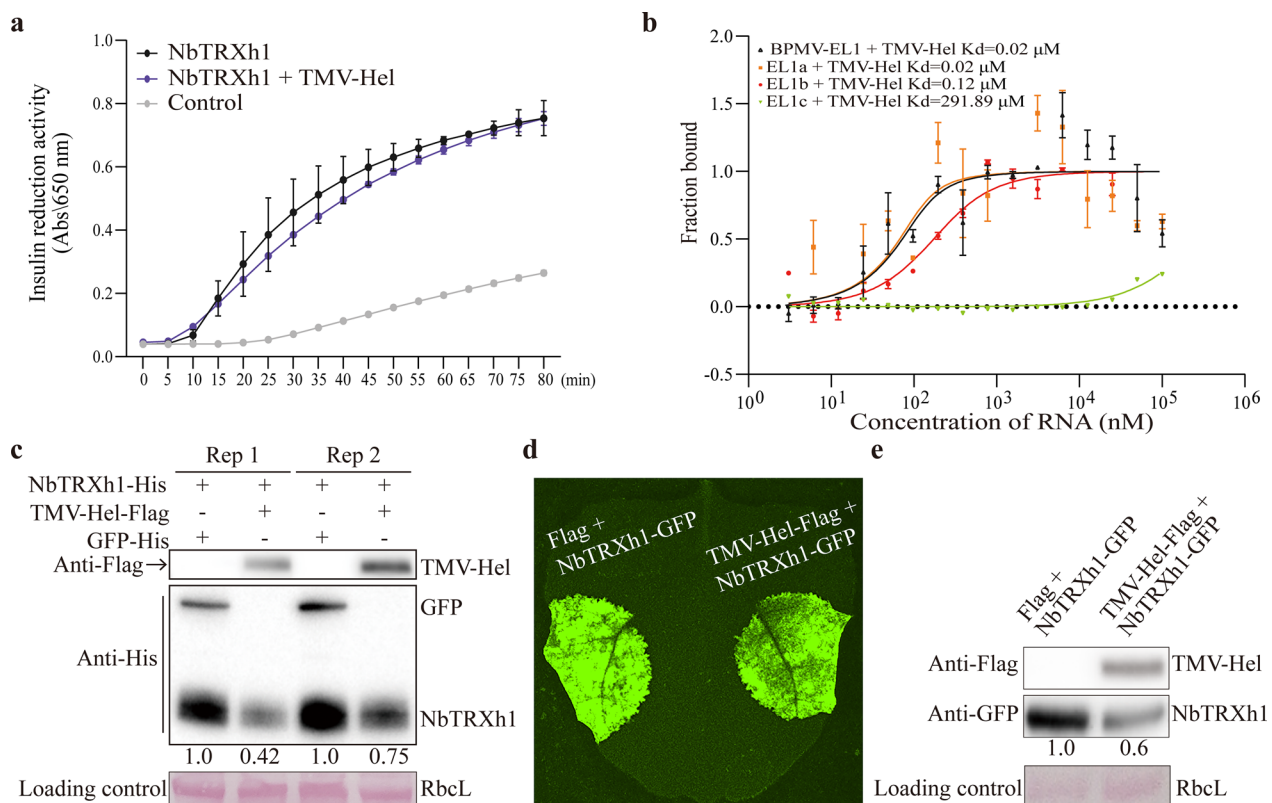


Fig. 7 The impact of TMV-Hel on NbTRXh1. **a** The reductase activity of NbTRXh1 was not influenced by adding TMV-Hel. In the reaction samples, 5 μM NbTRXh1 with or without TMV-Hel was subjected to insulin reduction activity assay. PBS buffer as a negative control. **b** Comparing the binding activities of TMV-Hel with BPMV-EL1 motif or its mutants of BPMV-EL1a-c by MST measurement. The sharply decreased binding value was occurred on EL1c-containing sample. Data are average $\pm$ SD, $n=3$. **c-e** In vivo detection of the effect of TMV-Hel binding to NbTRXh1 on the accumulation of NbTRXh1 in *N. benthamiana*. **c** Immunoassay of the accumulation of NbTRXh1 expressed from different treatments. Using half-leaf method, co-infiltration of *Agrobacterium* harboring NbTRXh1-His plasmid with that containing plasmids TMV-Hel-Flag or GFP-His (control). Two pools of half-leave samples (Rep 1, Rep 2), 9 leaves each pool, were collected for experimental assay at 5 dpi. Flag- and -His tag antibodies were used. **d, e** Using GFP as a detector in a set of co-infiltration NbTRXh1-GFP with TMV-Hel-Flag or pCambia1300-Flag (empty vector control). GFP image was captured with Fusion FX7 instrument (VILBER) and western blot to test NbTRXh1-GFP accumulation. In immunoassay analysis of fusion proteins (TMV-Hel-Flag, NbTRXh1-His, GFP-His or NbTRXh1-GFP), the tag antibodies of anti-Flag, -His or -GFP was used, respectively. The relative accumulation levels of NbTRXh1-His were quantified with ImageJ software. RbcL was served as a loading control. Three independent replicates of the experiment were performed

by examining the interaction with host/virus factors. BPMV-EL1 directly binds to a host immune response factor, NbTRXh1, specially at the conserved active site ⁴²WCGPC⁴⁶ (Mata-Perez et al. 2019), and suppresses its reductase activity, thereby revealing an important effect of a virus RNA motif on host enzymatic activity. While plant immune responding factor, NbTRXh1 involved in SA signaling pathway, exhibited a virus-induced expression, and overexpression of NbTRXh1 in *N. benthamiana* leaves caused a decreased GFP-expression, indicating an inhibited role of NbTRXh1 in CP-SGP activity.

In addition, virus helicase domain, TMV-Hel, binds to both the BPMV-EL1 motif and NbTRXh1. This is similar to TMV replicase attenuation of the NAC domain transcription factor ATAF2-induced SA-mediated

defense by interaction with and degradation of ATAF2 (Wang et al. 2009). The physical interaction between TMV-Hel and NbTRXh1 in *N. benthamiana* leaves resulted in a decrease in amount of NbTRXh1. Accordingly, the dual effects of BPMV-EL1 and TMV-Hel on the host NbTRXh1 enhanced the resistance of the virus against the host immune system. In addition to BPMV-EL1, corresponding tobamovirus EL1 motifs, the EL1 of other tobamoviruses tested such as TMV, hibiscus latent fort pierce virus (HLFPV), paprika mild mottle virus (PaMMV), tomato brown rugose fruit virus (ToBRFV), and zucchini green mottle mosaic virus (ZGMMV), also showed physical interaction with NbTRXh1 (Additional file 1: Figure S11), suggesting that the regulatory mechanism by which a virus RNA motif binds to a host defense

factor, such as NbTRXh1, may be conserved among tobamoviruses. Comparison of the binding activity and enzyme-activity-inhibition of BPMV-EL1 or TMV-EL1 on NbTRXh1 was also performed through EMSA and insulin reductase assay, respectively, and a relative stronger binding affinity of BPMV-EL1 was found, together with its correspondingly inhibitory effect on the redox activity of NbTRXh1 (Additional file 1: Figure S12a, b), which may explain at least partially its enhanced transcriptional activity.

Considering the vital role of secondary structure in SGP regulation, we also analyzed the secondary structure of BPMV-EL1 motifs with the RNAfold webserver (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). Comparing the predicted secondary structures of BPMV-EL1 and the BPMV-EL1a, BPMV-EL1b, and BPMV-EL1c mutants, four stem-loop structures (SLs) were folded by the sequence of BPMV-EL1, while no SL structures were formed by the BPMV-EL1a mutant, and only the lower and upper two SLs were formed in BPMV-EL1b and BPMV-EL1c, respectively (Additional file 1: Figure S13e). Considering the relative expression amounts of GFP from various constructs that included BPMV-EL1, BPMV-EL1a, BPMV-EL1b, or BPMV-EL1c (Additional file 1: Figure S13a-d), the presence of the upper two SLs in the secondary structure were indicated to be important. On the basis of the foregoing results, we assumed that the upper SLs may be correlated with GFP expression. Certainly, the tobamovirus-EL1 motif should only contribute a certain proportion to the activity of the full-length SGP.

Research on RNA sequence function and regulation has attracted substantial attention (Moore 2005). In addition, RNA virus genome-derived critical sequence contexts regulate interaction between the virus and host (Gallie et al. 1992; Tanguay et al. 1996), but the utility of the approach used might be limited. The methods currently used are complex owing to the involvement of gel mobility shift, ultraviolet-induced crosslinking assays, and sometimes isotopes are required (Marín-Béjar and Huarte 2015; McHugh et al. 2018; Zang and Lin 2016; Ying et al. 2024). Recently, an efficient in vivo method, RaPID, was developed to directly biotinylate proximal proteins associated with binding to specific RNA motifs in live cells (Ramanathan et al. 2018). Once the candidate RNA sequences/proteins have been obtained through the aforementioned methods, the RNA–protein interaction is often validated by the in vitro methods EMSA or MST. However, to the best of our knowledge, there is a paucity of in vivo detection methods. In this study, using the high binding activity of BoxB and λ N, as described by Ramanathan et al. (2018), we developed an in vivo RaPIDp method for screening potential proteins associated with

RNA elements of interest in plants. In RaPIDp, the RNA sequence of interest together with BoxB were integrated through agroinfiltration in the leaf of *N. benthamiana*, thus the potential binding proteins were located in living plant cells. We used BPMV-EL1 as a bait and identified NbTRXh1 acting as a host defense factor that interacted with BPMV-EL1. Based on our experimental application, the RaPIDp method is simple, fast, and effective for identification of candidate factors. In addition, BiFCrp, a method for in vivo detection of RNA–protein interactions, was established. The BiFCrp assay consists of three plant binary expression constructs to facilitate in vivo testing of RNA–protein binding activity. Furthermore, their subcellular location can be determined. Collectively, during assessment of tobamovirus EL1-interacting factors in *N. benthamiana*, we devised two in vivo techniques, RaPIDp and BiFCrp, to provide an efficient and convenient means for investigating the function of an RNA sequence of interest. The approaches are especially useful for the identification of functional RNA elements in plant cells.

Taken together, the present results revealed the molecular mechanism of an RNA virus replication strategy in plants. We have proposed a hypothetical working model illustrating a novel mechanism by which a viral SGP-related RNA motif combines with TMV-Hel in response to the host defense (Fig. 8). To achieve virus infection, a tobamovirus responds to the host through two closely related pathways: the BPMV-EL1b RNA motif binds to NbTRXh1 and suppresses its reducing activity, and the BPMV-EL1c RNA motif interacts with TMV-Hel to promote virus replication. In addition, TMV-Hel physically interacts with NbTRXh1, thereby suppressing the amount of NbTRXh1 protein in cells. Therefore, integration of these two mechanisms will accelerate the accumulation of sgRNA to facilitate virus replication and its survival in plant cells.

Conclusions

Virus regulatory elements, such as SGP, contribute significantly to the viral expression that associated with plant virus infection and the expression of exogenous proteins using viral vectors. In this study, we identified a host defense-related factor NbTRXh1 interacted with tobamovirus-EL1 RNA motif. In *N. benthamiana* transient expression system, EL1 and TMV-Hel superimpose their actions on NbTRXh1 leading to inactivated redox activity and reduced protein accumulation of NbTRXh1, revealing a mechanism used by tobamoviruses to combat plant immunity. The developed RaPIDp and BiFCrp methods also provide a practical tool for studying RNA–protein interactions specially applied to plant system.

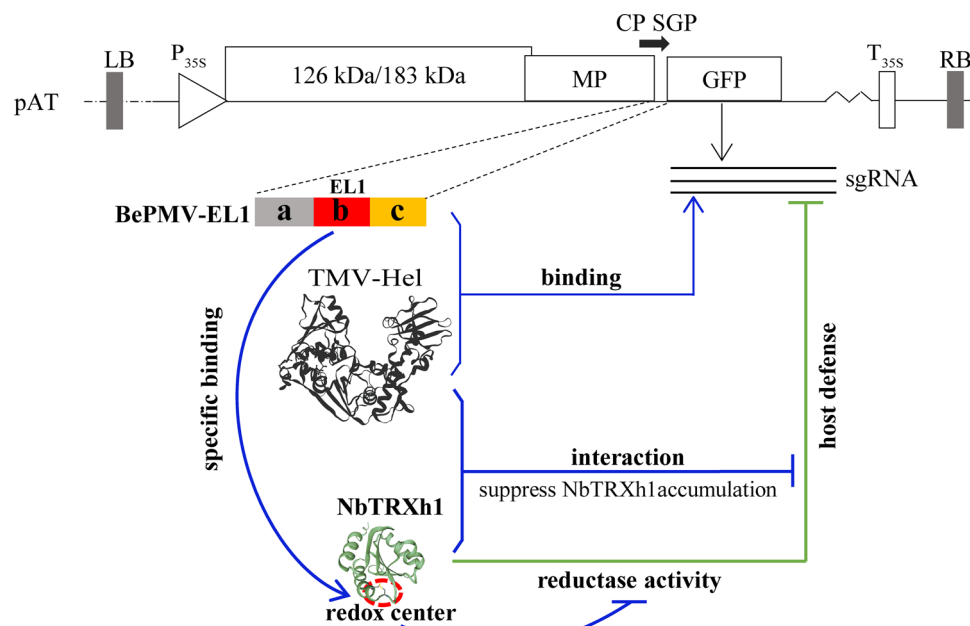


Fig. 8 The proposed interaction working model of virus BPMV-EL1 RNA motif, TMV-Hel domain, and host NbTRXh1. Using the pAT-derived constructs (as shown in Additional file 1: Figure S1a), the crucial motif of tobamovirus BPMV-EL1 (45 nt), divided by EL1a (grey), EL1b (red), and EL1c (yellow) regions, was identified. During the process of virus infection in *N. benthamiana* cells, host defense factor NbTRXh1 (represented in green), more specific its oxidative active site (⁴²WCGPC⁴⁶) (marked in red dotted circle), was first bound by BPMV-EL1b RNA motif, resulting a disruption of its redox activity. In addition, NbTRXh1 was interacted by viral protein TMV-Hel, inhibiting the amount of NbTRXh1 protein. Thus, tobamovirus EL1 and TMV-Hel superimpose their actions on NbTRXh1, revealing a mechanism used by tobamovirus in combating plant immunity

Methods

Plant culture

Nicotiana benthamiana plants were grown in a greenhouse (16 h light/8 h dark) at 23–24 °C. The leaves of 6-week-old plants were used for *Agrobacterium*-mediated transient expression.

Constructs

To generate the constructs for comparing the activity of recombinant SGPs from different *Tobamovirus* species, we used our previously reported pAT vector containing the GFP reporter gene driven by the TMV CP SGP (Huang et al. 2024), with the fragment (1126 bp) encoding Renilla luciferase (RiLuc) replaced GFP gene via *PacI/XhoI* digestion, and the pAT-Riluc plasmid was generated. The FiLuc expression cassette P_{NOS}-FiLuc-T_{NOS} was then further inserted into pAT-Riluc at the *PmeI* site obtaining the recombinant plasmid pAT-Dluc, in which the FiLuc expression was used as an internal control. To avoid contamination by the expression of the reporter gene from *Agrobacterium*, an intron was inserted in the ORF of the dual-luciferase reporter genes. To replace TMV-EL1 (45 nt located in the CP-ORF region, in which the ATG/AUG translation start codon was mutated to AGA), the intermediate vector pAT-ΔEL1-Dluc was generated by substitution of 45 nt of TMV-EL1 with

a synthetic fragment (310 nt) containing the *AfeI* site. Then, with a series of annealing primer pairs the corresponding sequence of different tobamovirus EL1 motifs (Additional file 2: Table S1, S2) was inserted in pAT-ΔEL1-Dluc by recombinant techniques (Vazyme Biotech Co., Ltd, ClonExpress-II One Step Cloning Kit, cat no. C112, Nanjing, China). The obtained series of recombinant plasmids, pAT-EL1n, were used for primary screening of the active EL1 motif. After the candidate chimeric SGPs with higher activity were identified, the original sequences of several potential EL1s replaced TMV CP SGP in pAT for further comparison and analysis. Finally, the recombinant plasmid pAT-BPMV-EL1-GFP was used for comparison of GFP expression using a half-leaf experimental method. In addition, pAT-BPMV-EL1-CP was generated for observation of virus symptoms in *N. benthamiana* (Additional file 1: Figure S5).

To investigate the binding activity of EL1 to different factors, the NbTRXh1 or TMV-Hel encoding sequence (369 nt and 1370 nt, respectively) was amplified with the specific primer pairs TRXh1-30-F/TRXh1-30-R and Hel-30-F/Hel-30-R (Additional file 2: Table S2) from cDNA of *N. benthamiana* and the pAT plasmid, respectively. The product was cloned into the pET-30a expression vector (at the *NdeI* and *NcoI/XhoI* sites, respectively) to obtain the recombinant plasmids pET30-NbTRXh1-His and

pET30-Hel-His, respectively. To generate the NbTRXh1_{SS} mutant, the NbTRXh1_{SS} mutant coding fragment (in which ⁴²WCGPC⁴⁶ was mutated to ⁴²WSGPS⁴⁶) was synthesized by the Universe Gene Technology (Tianjin) Co., Ltd and amplified with the primer pair TRXh1-30-F/TRXh1-30-R. The product was cloned into the pET-30a plasmid using a homologous recombination technique (Vazyme Biotech Co., Ltd, ClonExpress-II One Step Cloning Kit) to generate pET30-NbTRXh1_{SS}-His.

To investigate the effect of NbTRXh1 on virus replication and foreign protein expression, the NbTRXh1 over-expression plasmid (*NbTRXh1*-OE) was constructed, in which the cDNA fragment of NbTRXh1 (369 bp) was amplified by PCR with the primer pair 130-TH-F/130-TH-R and cloned into the plant expression vector pCambia1300-D4S (at the *Xba*I/*Sac*I sites) to yield p1300-NbTRXh1-His in which expression of NbTRXh1 was under the control of the CaMV 35S promoter. To knock-down NbTRXh1, an intron-spliced self-complementary construct *NbTRXh1*-RNAi that contains the sense and antisense sequences of the specific fragment (52–324 bp) of the NbTRXh1 cDNA region in the plant binary vector pCambia-HAN (Zhang et al. 2012) was generated. The sense and antisense sequences were amplified from *NbTRXh1* cDNA with the primer pairs TH-RiF1/TH-RiR1 and TH-RiF2/TH-RiR2, respectively. The sense fragment was cloned into the pCambia-HAN vector (at the *Eco*RI/*Kpn*I sites) to generate the intermediate vector Han-NbTRXh1, and the antisense fragment was cloned next to the intron spacer region of Han-NbTRXh1 (at the *Xba*I/*Bam*HI sites) to create the RNAi vector *NbTRXh1*-RNAi. To generate the constructs for subcellular localization experiments, the amplified eGFP/BFP fragment was cloned into p1300-NbTRXh1-His using a recombination method to obtain the constructs of p1300-NbTRXh1-eGFP and p1300-NbTRXh1-BFP respectively. The primers used in this study are listed in Additional file 2: Table S2, and all recombinant plasmids were verified by DNA sequencing.

Agrobacterium-mediated transient expression in *N. benthamiana*

Agrobacterium transformation was conducted as previously described (Huang et al. 2024). The binary vector was transformed into *Agrobacterium tumefaciens* strain GV3101. A single colony of *A. tumefaciens* containing different constructs was grown in LB liquid medium (supplemented with 30 mg/L rifampicin and 50 mg/L kanamycin at 28 °C for 24 h. An aliquot (200 µL) was transferred to 10 mL LB liquid medium [supplemented with 50 mg/L kanamycin, 10 mM 2-(4-morpholino)-ethane sulfonic acid, and 20 µM acetosyringone] and cultured overnight at 28 °C. The *A. tumefaciens* cells were

then harvested and resuspended with MMA infiltration buffer [10 mM MgCl₂, 10 mM 2-(4-morpholino)-ethane sulfonic acid, and 100 µM acetosyringone] to OD₆₀₀ = 1.0. After standing for 1 h at room temperature, agrobacterial cells were injected into the leaves of 6-week-old *N. benthamiana* plants with a needle-free syringe.

Western blot analysis

Western blot immunoassay was performed as reported previously (Huang et al. 2024). The antibody anti-GFP (Ray Antibody Biotech GFP-tag (ME11) Mouse Monoclonal Antibody, cat. no. RM1008, Beijing, China) or the antibody against TMV-CP (1:5000) prepared from mice via expression in *Escherichia coli* strain BL21 was used to probe the blot. The immunoblot signals were developed using the Western Blot Kit (SuperSignalTM West Dura Extended Duration Substrate, Thermo Scientific, cat. no. 34075, Rockford, IL, USA). Finally, the signal was detected with an Azure c600 gel imaging system (Azure Biosystems, Dublin, CA, USA).

Dual-luciferase assay

The RiLuc/FiLuc dual-luciferase assay was performed using the Dual-Glo[®] Luciferase Assay System Kit (Promega, Dual-Glo[®] Luciferase Assay System, cat. no. E2940, Madison, WI, USA) in accordance with the manufacturer's instructions.

RNA–protein interaction detection in plant agroinfiltration system (RaPIDp)

The RaPIDp method was developed based on the previously reported RaPID method (RNA–protein interaction detection) in living cells (HEK293T or neural progenitor cells) (Ramanathan et al. 2018), in which the RNA component comprised bacteriophage lambda BoxB stem-loops flanking the RNA motif of interest. As BoxB stem-loops bind to the 22-amino acid λN peptide with high affinity, the protein components associated with RNA-binding proteins may assemble around the λN-BirA fusion protein in which the λN peptide is fused to the N-terminus of BirA biotin ligase. In this manner, BoxB stem-loops recruit the RNA binding proteins, including the protein bound to the flanking region of the RNA motif of interest, and these proteins were biotinylated for further streptavidin capture and mass spectrometry.

To adapt to the plant agroinfiltration transient expression system, both the RNA and protein components in the RaPIDp method were constructed in a plant binary expression vector with certain modifications. Briefly, the synthetic fragment (220 bp) of the RNA component was cloned into the pCambia1300-derived vector p1300-GFP (*Spe*I) to obtain the p1300-Rmotif-GFP recombinant plasmid, in which the sequence of interest was

transcribed as the 3' UTR of GFP, and the single restriction site *AvrII*, *PmlI*, or *MluI* was designed for insertion of the target RNA sequence. The RNA sequence EL1 was inserted at the *PmlI* site of p1300-Rmotif-GFP by a homologous recombination method to obtain p1300-EL1-GFP. The synthesized fragment (1032 bp) encoding the λ N-BirA fusion protein was cloned into p1300-GFP (at the *XbaI/SalI* sites) to obtain the recombinant p1300- λ N-BirA, in which GFP was replaced by the fusion protein λ N-BirA (BASU from *Bacillus subtilis* was used as the biotin ligase). To aid in the cytoplasmic expression of the fusion protein, a nuclear exit signal peptide from *Arabidopsis* Tubulin (NES), together with the HA tag, was added to the fusion protein.

The *Agrobacterium* combination of p1300-BPMV-EL1-GFP with p1300- λ N-BirA was co-agroinfiltrated into *N. benthamiana* leaves. The combination of p1300-Rmotif-GFP with p1300- λ N-BirA was used as a negative control. The plant was cultured for 3–7 d in a greenhouse for leaf sampling and protein extraction. Biotinylated proteins were captured by Invitrogen™ Dynabeads™ MyOne™ Streptavidin C1 (Thermo Fisher Scientific, cat. no. 65002, Baltics, UAB). Briefly, the extracted total proteins were bound to the beads pre-coated with coating buffer (0.1 M sodium borate buffer, pH 9.5), then incubated with rotation at 37 °C for 16–24 h to capture the proteins. The tube was placed on the magnet for 2 min and the supernatant was discarded, then the beads were washed three times with wash buffer [PBS (pH 7.4), 0.1% bovine serum albumin, and 0.05% Tween® 20]. Finally, the biotinylated protein sample was eluted with 100 μ L elution buffer (0.1 M glycine-HCl, pH 2.5) and subjected to mass spectrometric analysis.

Bimolecular fluorescence complementation assay for RNA-protein interaction (BiFCrp)

Based on the principle of the BiFC technique (Yu et al. 2023), the high binding activity of the λ N peptide to BoxB stem-loops was utilized to develop the BiFCrp method for in vivo detection of the binding activity between the RNA sequence BPMV-EL1 and its target protein NbTRXh1. The BiFCrp assay used the constructs 1300-Rmotif-RiL, 1300-YFPn- λ N, and 1300-Factor-YFPc. The 1300-Rmotif-RiL vector was constructed to avoid GFP-fluorescence noise by replacing GFP-ORF with RiLuc. The 1300-BPMV-EL1-RiL vector contained BPMV-EL1 flanked by BoxB stem-loops. To enable the target RNA motif to link with YFPn, an accessory vector 1300-YFPn- λ N was designed, in which the λ N peptide was fused with the split YFPn. Given the high affinity of λ N with BoxB, the target RNA was bundled together with BoxB and YFPn- λ N (as shown in Fig. 2a). The vector 1300-Factor-YFPc harbored the candidate factor

NbTRXh1 fused with the split YFPc. These three plasmids were transferred separately into *A. tumefaciens* strain GV3101 followed by co-infiltration into *N. benthamiana* leaves. The *agrobacteria* harboring different plasmids (1300-Rmotif-RiL, 1300-YFPn- λ N, or 1300-Factor-YFPc) were mixed in the ratio of 1:1:1 (v/v/v) at OD₆₀₀=0.8. At 2 days post-agroinfiltration, the YFP signal in the leaf sample was visualized under a fluorescence microscope. If the target RNA, such as BPMV-EL1, could interact with the host factor (NbTRXh1), the assembled split YFPn and YFPc that are in close proximity will emit yellow fluorescence. The *Agrobacterium* mixture containing the 1300-Rmotif-RiL plasmid lacking the target RNA, or the 1300-YFPc empty vector was used as a negative control.

Confocal laser scanning microscopy

Leaf samples were observed with a Zeiss LSP8 confocal microscope. The GFP signal was visualized at 488 nm and that of YFP at 514 nm.

Electrophoretic mobility shift assay (EMSA)

The fusion protein NbTRXh1-His, NbTRXh1ss-His, or TMV-HEL-His was expressed from pET-30a in *E. coli* strain BL21 (DE3) pLysS under 0.5 mM IPTG at 16 °C overnight. The recombinant protein purified with Ni Sepharose™ excel (GE Healthcare, Uppsala, Sweden, cat. no. 17–3712-02) was applied to EMSA with the Chemiluminescent Nucleic Acid Detection Module Kit (Prod. # 89,880, Thermo Fisher Scientific, Rockford, IL, USA). The BPMV-EL1 and its related mutant RNA probes were synthesized and labeled with biotin by Beijing Tsingke Biotech Co., Ltd. The unlabeled probe was used as a competitive cold probe. The GFP-His fusion protein was used as a negative control.

MicroScale thermophoresis assay

The RNA-protein binding experiments were performed with a Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany) in accordance with a previously reported method (Wang et al. 2020) with some modifications. The recombinant proteins NbTRXh1-His and TMV-Hel-His were labelled with RED-dye. The synthesized BPMV-EL1 RNA fragment (45 nt) by the Beijing Tsingke Biotech Co., Ltd. was used as a ligand. The labelled NbTRXh1-His or TMV-Hel-His together with 16 titrations of BPMV-EL1 RNA were loaded onto standard treated silicon capillaries (MO-K002 Monolith™ NT.115; NanoTemper Technologies) and the fluorescence was measured. The data were analyzed with the software MO. Affinity Analysis v2.2.4.

Insulin reduction assay

The insulin reduction assay was performed as previously described (Holmgren 1979) with slight modification. The mixture of 5 μ M fusion protein NbTRXh1-His or NbTRXh1ss-His with the synthetic RNA was added to the reaction buffer [0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.13 mM bovine insulin, and 0.33 mM DTT]. PBS buffer was used as a negative control. The reduction activity was monitored at 650 nm at room temperature with a spectrophotometer.

Pull-down assay

The in vitro interaction of TMV-Hel and NbTRXh1 was analyzed by conducting a glutathione *S*-transferase (GST) pull-down assay. The full-length coding sequence of NbTRXh1 (369 bp) or NbTRXh1ss amplified by PCR with the primer pair TH-GEX-F/TH-GEX-R was cloned into the pGEX vector to obtain pGEX-NbTRXh1 or pGEX-NbTRXh1ss. The purified fusion protein NbTRXh1-GST, NbTRXh1ss-GST, or GST was incubated with TMV-Hel-His separately. After immunoprecipitation with glutathione-sepharose beads, the sample was detected by western blotting with anti-His or anti-GST antibodies, respectively.

Reverse-transcription quantitative PCR (RT-qPCR)

The 0.1 g leaf sample was ground in liquid nitrogen and total RNA was extracted using the TRIzol Reagent (Invitrogen, cat. no. 15596–018, Carlsbad, CA, USA). The quality of the isolated RNA was measured with a spectrophotometer, then reverse transcription was performed with the Vazyme SuperScript™ III First Strand Synthesis System (cat. no. R323-01) to synthesize the cDNAs. The RT-qPCR analyses were performed with the Taq Pro Universal SYBR qPCR Master Mix (Vazyme, cat. no. Q712-02) to determine the relative expression levels of *NbTRXh1*. Primers used for RT-qPCR are listed in Additional file 2: Table S2. The *N. benthamiana* *ACTIN* gene was used as an internal control.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test in the GraphPad Prism9 software.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-025-00365-x>.

Additional file 1: Figure S1. Mining of active EL1 elements from tobamoviruses. **Figure S2.** In vivo verify the BiFCrp method by testing the interaction of TMV UPD with EF1A. **Figure S3.** Transcriptional- and protein-level analyses of the constructs in BiFC and BiFCrp related experiments. **Figure S4.** In vivo detecting the interaction of BPMV-EL1 with NbTRXh1 by BiFCrp. **Figure S5.** Effect of *NbTRXh1*-OE or -RNAi prior to pAT-BPMV-EL1-CP-inoculation on virus symptom in *N. benthamiana*. **Figure S6.**

TMV-induced expression of NbTRXh1 and its subcellular localization. **Figure S7.** BPMV-EL1b and the redox site of NbTRXh1 are crucial for their interaction. **Figure S8.** The interaction of TMV-Hel with both BPMV-EL1 motif and NbTRXh1. **Figure S9.** The influence of TMV-Hel on GFP-expression. **Figure S10.** No effect of NbTRXh1 was detected on the accumulation of TMV-Hel. **Figure S11.** RNA-EMSA assay of the binding activity of NbTRXh1 to different tobamovirus-EL1 motifs. **Figure S12.** Comparative analysis of BPMV-EL1 and TMV-EL1 motifs on NbTRXh1 activity. **Figure S13.** Comparative analysis of the activities of BPMV-EL1 and its mutants BPMV-EL1a-c in viral GFP-expression.

Additional file 2: Table S1. The sequences of EL1 motifs from various Tobamovirus species. **Table S2.** The primer used in this study. **Table S3.** Screened factors potentially interacted with BPMV-EL1.

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Author contributions

YZ and RF conceived the study; YW, WH, and NX performed the experiments; YW and YZ performed data analysis and wrote the manuscript with contributions from all the authors; YZ and RF supervised the experiments and revised the manuscript.

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Data availability

The datasets used and analyzed during the current 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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