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Bacterially Expressed Double-Stranded RNAs against Hot-Spot Sequences of Tobacco Mosaic Virus or Potato Virus Y Genome Have Different Ability to Protect Tobacco from Viral Infection

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Abstract Posttranscriptional gene silencing, also known as RNA interference, involves degradation of homologous mRNA sequences in organisms. In plants, posttranscriptional gene silencing is part of a defense mechanism against virus infection, and double-stranded RNA is the pivotal factor that induces gene silencing. In this paper, we got seven hairpin RNAs (hpRNAs) constructs against different hot-spot sequences of Tobacco mosaic virus (TMV) or Potato virus Y (PVY) genome. After expression in *Escherichia coli* HT115, we extracted the seven hpRNAs for the test in tobacco against TMV or PVY infection. The data suggest that different hpRNAs against different hot-spot sequences of TMV or PVY genome had different ability to protect tobacco plants from viral infection. The resistance to TMV conferred by the hpRNA against the TMV movement protein was stronger than other TMV hpRNAs; the resistance to PVY conferred by the hpRNA against the PVY nuclear inclusion b was better than that induced by any other PVY hpRNAs. Northern blotting of siRNA showed that the resistance was indeed an RNA-mediated virus resistance.

Keywords Tobacco mosaic virus · Potato virus Y · RNA interference · hpRNAs · Hot-spot sequences

Introduction

Tobacco mosaic virus (TMV) and Potato virus Y (PVY) are major plant diseases that cause serious crop losses worldwide [1–3]. The prevention of plant viruses always uses chemical pesticides, which cause chemical residue accumulation in plants and reduce their quality. In

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1986, Beachy first transformed the coat protein gene (CP) of TMV into tobacco and achieved the first transgenic tobacco in the world. Later, scientists transformed different genes or complement DNAs (cDNAs) derived from different viruses into plants, which acquired the corresponding resistance [4–11]. Initially, researchers thought that the resistance might function at a protein level [7], but subsequent studies showed that the resistance was at the RNA level. In fact, it was posttranscriptional gene silencing (PTGS) mediated by double-stranded RNA (dsRNA) and induced by small interference RNA (siRNA) [6, 8, 9]. Originally, the construction of hairpin RNAs (hpRNAs) derived from gene sequences of viruses and their transformation into plants, to produce transgenic virus-resistant plants, was performed as an economical and effective way against virus infection. However, because of the risks of transgenic plants to ecological safety, the spreading of those plants has been greatly restricted in practice. In 2001, Timmons et al. [12] discovered that a bacterial strain lacking the dsRNA-specific endonuclease RNaseIII could be cultured to produce high levels of specific dsRNAs, and those dsRNAs could effectively trigger strong and gene-specific gene silencing when fed to *Caenorhabditis elegans*. Later, Tenllado et al. [13] tested this method for the control of plant viruses and achieved positive results.

TMV is a model plant virus that is studied and sequenced first. It has four different functional genes: replicase protein (RP), RNA polymerase protein (RNA), movement protein (MP), and CP [14]. The viral genome of PVY contains one long open reading frame (ORF) expressed as a polyprotein precursor. This polyprotein undergoes proteolytic processing to yield about seven smaller proteins denoted as helper component protein (HC-Pro), nuclear inclusion b (Nib), CP, and so on [15]. These genes have different functions in the replication and infection processes of plant viruses [16, 17]. In this study, we compared the local resistance of different hpRNAs derived from the hot-spot sequences of TMV or PVY genome via *Escherichia coli* HT115. We discovered that the hpRNAs from the TMV MP and the PVY NIb hot-spot sequences had stronger resistance. The hpRNA technology is a useful and effective strategy for protecting plants against virus infection, and this study should take up a prominent position in both fundamental and applied research.

Materials and Methods

Polymerase Chain Reaction Primers

Table 1 lists all primers used in this study. The different cDNA fragments of TMV or PVY were amplified by reverse transcriptase polymerase chain reaction (PCR) and linked into the pGEM-T Easy vector (Promega, Beijing, China) for sequence analysis. The accession numbers for TMV were EU796224, EU796225, DQ014551, and EU796226 representing the RP gene, the MP gene, the CP gene, and the RNA gene, respectively. The accession number of the PVY sequence was EU182576.

Bacterial Strains, Plasmids, Media, Chemicals, and Other Reagents

Table 2 lists bacterial strains and plasmids used in this work. *Taq* DNA polymerase, agarose, and other chemicals came from TaKaRa (Dalian, China). KOD-plus DNA polymerase was from Toyobo Co., Ltd. (Tokyo, Japan). Lysogeny broth (LB), SOB, and SOC medium were prepared as described in Sambrook and Russell [18]. *Taq* DNA

Table 1 PCR primers.

Primers	Primer sequence (5′→3′)[a]	Specific use
TMV CPI-5	GCGGGCCC ATGTCTTACAGTATCACTAC	Construction of TMV pGEM-CP480
TMV CPI-3	GCGCGAATTCTCAAGTTGCAGGACCAGAG	Construction of TMV pGEM-CP480
TMV CPII-5	GCGCCTGCAGTCAAGTTGCAGGACCAGAG	Construction of TMV pGEM-CP480
TMV CPII-3	GCGCGTCGAC ATGTCTTACAGTATCACTAC	Construction of TMV pGEM-CP480
GUSII-5	GCGCGAATTCGCAGATGACAGTGGCATCG	Amplification the glucuronidase gene of plasmid pBI121
GUSII-3	GCGCCTGCAGGACTGCCTCTTCGCTGTAC	Amplification the glucuronidase gene of plasmid pBI121
TMV MPI-5	GCGCGGGCCCTTTAGCCGGTTTGGTCGTCAC	Construction of TMV pGEM-MP480
TMV MPI-3	GCGCGAATTCTTACTACTATTTTTCCCTTTGCG	Construction of TMV pGEM-MP480
TMV MPII-5	GCGCCTGCAGTTACTACTATTTTTCCCTTTGCG	Construction of TMV pGEM-MP480
TMV MPII-3	GCGTCGACTTTAGCCGGTTTGGTCGTCAC	Construction of TMV pGEM-MP480
TMV RNAI-5	GCGCGGGCCCTTCGATCCAAACGGAGTACCC	Construction of TMV pGEM-RNA480
TMV RNAI-3	GCGCGAATTCTTGATTATTTTCTCCATCGGAAG	Construction of TMV pGEM-RNA480
TMV RNAII-5	GCGCGTCGACTTGATTATTTTCTCCATCGGAAG	Construction of TMV pGEM-RNA480
TMV RNAII-3	GGGAATTCCATATGTTCGATCCAAACGGAGTACCC	Construction of TMV pGEM-RNA480
TMV RPI-5	GCGCGGGCCCTGGGAACGCATTTCCCTCCG	Construction of TMV pGEM-RP480
TMV RPI-3	GCGCGAATTCTCTCCTCTGGCCATCGAAC	Construction of TMV pGEM-RP480
TMV RPII-5	GCGCCTGCAGTCTCCTCTGGCCATCGAAC	Construction of TMV pGEM-RP480
TMV RPII-3	GCGCGAGCTCTGGGAACGCATTTCCCTCCG	Construction of TMV pGEM-RP480
PVYHC-ProI-5	GCGCGGGCCCGAAAGGAAAGGAAAATACAGC	Construction of PVY pGEM-HcPro600
PVY HC-Pro I-3	GCGCCCGCGGCCTTGCCTGGCAATATATAAC	Construction of PVY pGEM-HcPro600
PVYHC-Pro II-5	GCGCGTCGACGAAAGGAAAGGAAAATACAGC	Construction of PVY pGEM-HcPro6000
PVYHC-Pro II-3	GCGCCTGCAGCCTTGCCTGGCAATATATAAC	Construction of PVY pGEM-HcPro600
PVY NIb I-5	GCGCGGGCCCGCGATTGTATAAAGGCTTGC	Construction of PVY pGEM-Nib600
PVY NIb I-3	GCGCGAATTCGCAATCAGCAGATCATCACC	Construction of PVY pGEM-Nib600

Table 1 (continued).

Primers	Primer sequence (5′→3′)[a]	Specific use
PVY NIb II-5	GCGCGTCGACGCGATTGTATAAAGGCTTGC	Construction of PVY pGEM-Nib600
PVY NIb II-3	GCGCCTGCAGGCAATCAGCAGATCATCACC	Construction of PVY pGEM-Nib600
PVY CP I-5	GCGCGGGCCCGTGAATGTTGGAACATCTGG	Construction of PVY pGEM-CP600
PVY CP I-3	GCGCGAATTCGAAAAGTCGAGATTGAGCTG	Construction of PVY pGEM-CP600
PVY CP II -5	GCGCGTCGACGTGAATGTTGGAACATCTGG	Construction of PVY pGEM-CP600
PVY CP II -3	GCGCCTGCAGGAAAAGTCGAGATTGAGCTG	Construction of PVY pGEM-CP600
M13F-47	CGCCAGGGTTTTCCCAGTCACGAC	Identification of different vectors
M13R-48	AGCGGATAACAATTTCACACAGGA	Identification of different vectors

[a] The underlined sequences are different restriction endonuclease sites

polymerase was used in all PCR tests. KOD-plus DNA polymerase was used to generate DNA fragments for cloning. TIANGEN products (TIANGEN Biotech Co., Ltd., Beijing, China) were used to isolate plasmid DNAs, gel-purify fragments, or purify PCR products. The concentration and purity of all PCR products, extracted dsRNA, and DNA were determined with a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). We used the North Carolina 89 (NC89) tobacco lines for the test against viral infection.

Table 2 *E. coli* strains and plasmids used in this study.

Strains or plasmids	Genotype/properties/specific use	Source/Reference
HT115	F-mcrA mcrB IN (rrnD-rrnE) 1, rnc14::Tn10(DE_3 lysogen:lacUV5 promoter-T7 polymerase), used for the production of ihpRNAs, tet^R	Dasgupta et al. [19], Takiff et al. [20]
DH5α	F^-, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1, used for subcloning recombinant plasmids	Sambrook and Russell [18]
pBI121	Used as a template for amplifying a 120-bp glucuronidase gene, amp^R	Chen et al. [21], AF485783
pGEM-T Easy	Cyclized at the *Eco*RI site and used for the construction of different vectors, amp^R	Promega
TMVCP,TMVMP, TMVRP, TMVRNA, PVYHCPro, PVYCP, PVYNIb	Used for hpRNA production, amp^R	This study

tet^R tetracycline resistance gene, amp^R ampicillin resistance gene

Construction of Different Vectors for Different hpRNAs

In this paper, we constructed seven hpRNA vectors against the TMV MP gene (from nt 301 to nt 780), CP gene (from nt 1 to nt 480), RP gene (from nt 301 to nt 780), RNA gene (from nt 301 to nt 780), PVY HC-Pro gene (from nt 372 to nt 972), NIb gene (from nt 471 to nt 1071), CP gene (from nt 100 to nt 700). PCR reactions were carried out in the Eppendorf Mastercycler in 50 μL volume containing 1 U of KOD-plus DNA polymerase. The PCR products were ethanol precipitated and resuspended in appropriated TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5). Afterwards, PCR products were size-fractionated on a 1% agarose gel in TAE (40 mmol/L Tris base, 1.142 mL/L acetic, 0.01 mol/ L EDTA, pH 8.0) buffer. The corresponding band was excised and purified with a TIANGEN Midi Purification Kit according to the manufacturer's protocol, except that in the last step the amplified linear DNAs were quantified by spectroscopy. The details of different vectors were described in Fig. 1 (taking TMV pGEM-MP as an example). For TMV, we subcloned a 480-bp TMV MP gene as the target sequence of the hpRNA and subcloned a 120-bp glucuronidase (GUS)

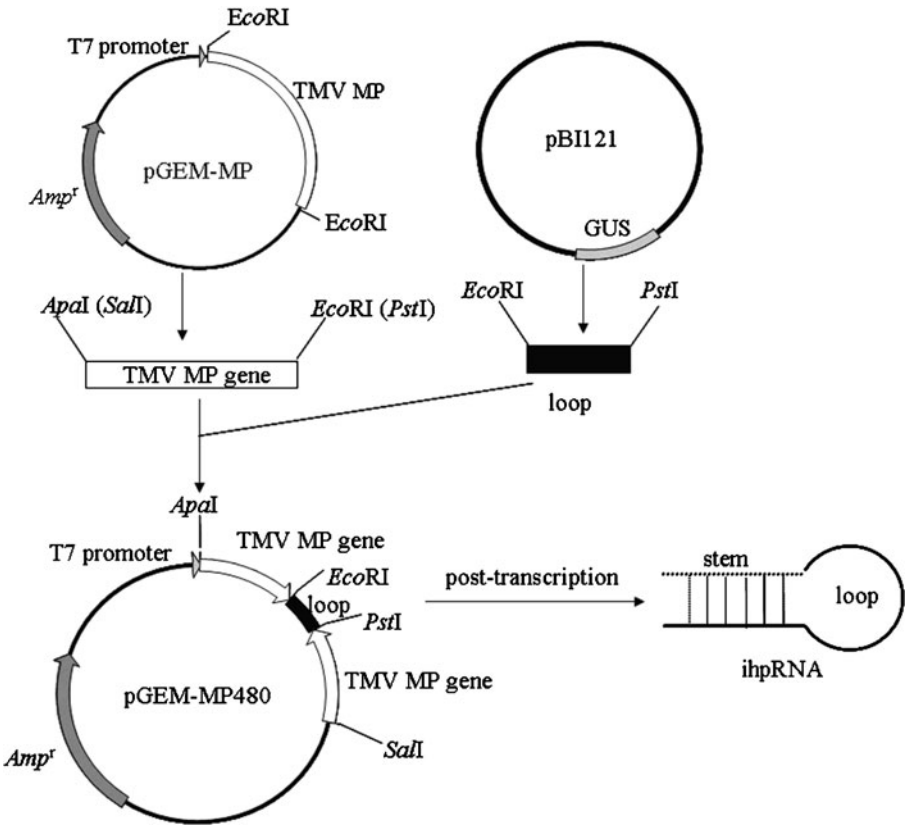

Fig. 1 The construction of hpRNA vectors. The construction process included PCR amplification, transformation, and posttranscription. pBI121 vector was used as a template for amplifying a 120-bp glucuronidase gene, amp^R pGEM-MP480 vector, one T7 promoter, inverted-repeat part of TMV cDNA sequence

gene as the spacer region of the hpRNA. For PVY, we subcloned a 600-bp PVY CP gene as the target sequence of the hpRNA and subcloned a 120-bp GUS gene as the spacer region of the hpRNA.

Colony-direct PCR (CD-PCR) was used to show that all vectors of hpRNAs were correct. The PCR reactions were performed by using the specific primers of the pGEM-T Easy vector (M13F-47 and M13R-48) to test for the new junction fragments. The positive and negative control colonies were always tested side-by-side. All PCR products were analyzed by 1% agarose gel.

Induction and Extraction of hpRNAs

All correct vectors containing hpRNAs were transformed into *E. coli* strain HT115. Single colonies were grown for 16 h in LB with 100 μg/mL ampicillin and 12.5 μg/mL tetracycline at 37°C. The culture was diluted 50-fold in 10 mL of the same medium and allowed to grow to OD600 of 0.6–0.8. T7 RNA polymerase was induced by the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside, and the culture was incubated with shaking for 4 h at 37°C. TRIZOL Reagent (Invitrogen, Carlsbad, CA) was used for the isolation of total RNA from bacteria, following the instructions of the supplier. The nucleic acid pellet was resuspended in TE buffer and used for 1% agarose gel electrophoresis. Accumulation of hpRNAs in bacterial extracts were confirmed by treatment with 100 μg/mL RNase A under high salt conditions (0.3 mol/L NaCl, 0.03 mol/L sodium citrate) for 37°C, 1 h. Then, the dsRNAs were purified by gel-extraction, and reverse transcriptase PCR plus sequencing was done to make sure that the expressed dsRNAs have correct sequences. The correct strains were used to produce hpRNAs according to the method of Tenllado et al. [13].

Local Resistance Analysis of the Extracted hpRNA Transcripts

For comparison, we tested 50 tobacco plants at the four-leaf stage for each vector (in three independent experiments). The TMV/pGEM or PVY/pGEM (inoculated with the total RNA extracted from the pGEM-T plasmid and TMV or PVY inoculum) and hpRNA (derived from the heterogenous vector of PVY CP or TMV CP) plants were used as control. For each tested plant, about 100 μg of hpRNA was applied, regardless of the vector used for hpRNA production. The titers of TMV and PVY were 2 and 3 μg/mL of virus sap, respectively. The plants were inoculated with equal volume hpRNAs (100 μL per tobacco leaf) of TMV (or PVY) produced by the different vectors. Enzyme-linked immunosorbent assay detection and local resistance analysis were carried out according to Liu et al. [22].

Northern Blotting

The total RNA and the small RNA of inoculated tobacco leaves were extracted using RNeasy Mini Kit (TIANGEN Biotech Co., Ltd.) and PureLink miRNA Isolation Kit (Invitrogen), respectively, according to the instructions of the supplier. Electrophoresis, blotting, and EDC cross-linking of small RNA fractions were carried out sequentially according to Pall et al. [23]. Probe synthesis, siRNA hybridization, and chemiluminescence detection (DIG Northern Starter Kit, Roche, Germany) were performed according to Goto et al. [24]. The probes of viral siRNAs were the same to the ones of viral total RNA.

Results

Construction of Different Vectors and Extraction of hpRNAs

For comparison, the different vectors were designed to produce the hpRNAs containing TMV or PVY cDNA sequences under the control of the T7 promoter. To simplify the descriptions of the bacterial strains and vectors used in this study, we used the following terminology: HT115 is an RNase III-deficient bacterial host harboring a λDE3 lysogen (source of T7 polymerase), and TMV CP is a plasmid that could produce the hpRNAs of the TMV CP gene. TMV RP, TMV MP, TMV RNA, PVY HC-Pro, PVY Nib, and PVY CP are named analogously to the plasmid TMV CP. These vectors were transformed into HT115 and used for the hpRNA production. Inducing the T7 promoter with isopropyl β-D-1-thiogalactopyranoside, we found that all strains harboring the above plasmids accumulated significantly higher levels of hpRNAs, while the accumulation of hpRNAs by the RNaseIII-expressing strains was not detectable by gel electrophoresis (Fig. 2). We also compared the RNA samples extracted from *E. coli* before and after the treatment with RNase A (Fig. 3). After treatment with RNase A, the dsRNA bands were still existed, indicating that what we extracted were dsRNAs. Sequencing results also showed that the dsRNAs are correct.

Local Resistance Analysis of the Extracted hpRNAs

At 14 days postinoculation (dpi), all wild-type tobacco showed typical TMV mosaic or PVY vein necrosis on their leaves. The symptoms of the tested tobacco plants were assessed approximately every 10 days. The final classification refers to the state of the plants after the last assessment date. We discerned two major categories of plants after simultaneous treatment of TMV (or PVY) and the bacterial hpRNAs: local resistant and susceptible. The susceptible tobacco showed varying degrees of symptoms, some had very mild symptoms (very mild green mosaicism compared with the TMV-infected control tobacco or light vein clearing with the PVY-infected control tobacco). The local resistant plants remained

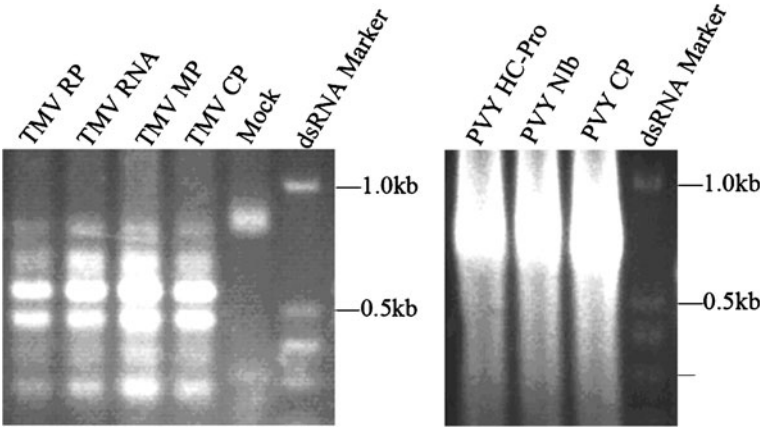

Fig. 2 hpRNAs produced by different vectors. Mock: the DH5α harboring the TMVpGEM-CP480 plasmid. TMVCP, TMVMP, TMVRP, TMVRNA, PVYHCPro, PVYCP, and PVYNIb were used for hpRNA production. The dsRNA band produced by TMV RP, TMV RNA, TMV MP, and TMV CP was 480 bp. The dsRNA band produced by PVY HC-Pro, PVY NIb, and PVY CP was 600 bp

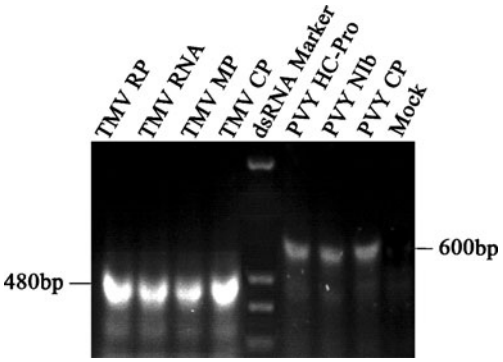

Fig. 3 the RNA samples extracted from *E. coli* after the treatment with RNase A. After treatment with RNase A, the purpose dsRNA bands were still existed, indicating that what we extracted were dsRNAs. The dsRNA band produced by TMV RP, TMV RNA, TMV MP, and TMV CP was 480 bp. The dsRNA band produced by PVY HC-Pro, PVY NIb, and PVY CP was 600 bp

symptom-free, even beyond 2 months postinoculation. The hpRNAs derived from the hot-spot sequences of TMV or PVY genome could all protect tobacco from TMV or PVY infections. The local resistance percentages were obviously different from each other (Tables 3 and 4). The resistance to TMV conferred by the hpRNA against the TMV MP was stronger than other TMV hpRNAs; the resistance to PVY conferred by the hpRNA against the PVY Nib was better than that induced by any other PVY hpRNAs.

Table 3 Interference with different TMV hpRNAs transcripts.

hpRNAs preparation	Phenotype[a]	Resistant percentage (%)	Average resistant ratio (%)[b]
pGEM	0/10	0.00	0.00
	0/10	0.00	
	0/10	0.00	
PVY CP	0/10	0.00	0.00
	0/10	0.00	
	0/10	0.00	
TMV CP	9/18	47.06	47.91±1.8219d
	7/15	46.67	
	8/17	50.00	
TMV MP	13/19	68.75	65.72±4.9596c
	9/15	60.00	
	11/16	68.42	
TMV RP	7/20	40.00	33.89±6.7358a
	4/15	26.67	
	6/15	35.00	
TMV RNA	7/16	42.11	39.73±5.6000b
	5/15	33.33	
	8/19	43.75	

[a] The number of plants showing resistant penotypes to TMV infection is indicated

[b] Different letters indicate significant differences between treatments at $P<0.05$ (Duncan's multiple range test)

Table 4 Interference with different PVY hpRNAs transcripts.

hpRNAs preparation	Phenotype[a]	Resistant percentage (%)	Average resistant ratio (%)[b]
pGEM	0/10	0.00	0.00
	0/10	0.00	
	0/10	0.00	
TMV CP	0/10	0.00	0.00
	0/10	0.00	
	0/10	0.00	
PVY CP	8/15	54.55	56.47±4.4290b
	8/13	61.54	
	12/22	53.33	
PVY NIb	14/18	66.67	71.68±5.6351a
	12/17	70.59	
	10/15	77.78	
PVY HC-Pro	7/15	47.62	45.71±2.5198c
	6/14	42.86	
	10/21	46.67	

[a] The number of plants showing resistant penotypes to PVY infection is indicated

[b] Different letters indicate significant differences between treatments at $P<0.05$ (Duncan's multiple range test)

In all cases, the TMV- or PVY-enzyme-linked immunosorbent assay values of local resistant tobacco showed no great difference from those of the negative control tobacco, and the virus content in the susceptible tobacco was clearly lower than in the positive control tobacco, but higher than in the negative control tobacco (Tables 5 and 6 showed only a partial listing of plants in test results).

Northern Blotting

Virus resistance in plants containing a virus-derived transgene is caused by posttranscriptional gene silencing of the transgene [25–27]. To prove whether the exogenous hpRNAs could induce plant local resistance, we investigated the presence or absence of virus RNA and siRNA in tested tobacco plants at the end of the experiment. We used the TMV/pGEM or PVY/pGEM and pGEM/Mock of the same age as positive and negative controls. TMV or PVY RNA signals were detected in the susceptible tobacco but not in the resistant tobacco and pGEM/Mock tobacco (Fig. 4). Northern blot results demonstrating a decreased accumulation of TMV or PVY RNA in tested local resistant tobacco. We detected siRNA signals in the resistant and susceptible tobacco, but not in the wild-type tobacco (Fig. 5).

Discussion

Compared with other approaches in antiviral transgenic engineering, RNA-mediated virus resistance (RMVR) is highly efficient (yielding almost complete immunity), enduring, and highly biosafe [22, 25, 27–29]. Recent studies have shown that bacterially produced dsRNA could interfere with virus infection [13, 30–32]. As a means of acquiring transgenic plants, using dsRNA transcripts provided by this strategy for RNA interference has

Table 5 ELISA detection of TMV in some tobacco plants interference with different TMV hpRNAs transcripts ($OD_{490\ nm}$)[a].

TMV hpRNA	No. of tobacco plant	Phenotype	Results of ELISA	TMV hpRNA	No. of tobacco plant	Phenotype	Results of ELISA
RP	7	R	0.094±0007	RNA	13	R	0.092±0.002
	18	R	0.090±0.008		32	R	0.088±0.011
	43	R	0.095±0.012		47	R	0.089±0.006
	14	S	0.390±0.009		17	S	0.382±0.008
	35	S	0.395±0.004		29	S	0.380±0.007
	38	S	0.389±0.008		42	S	0.373±0.004
CP	18	R	0.091±0.001	MP	8	R	0.081±0.006
	37	R	0.092±0.004		13	R	0.088±0.005
	48	R	0.087±0.002		34	R	0.087±0.002
	9	S	0.391±0.003		23	S	0.392±0.005
	23	S	0.386±0.012		34	S	0.402±0.006
	35	S	0.399±0.001		39	S	0.396±0.008
TMV/pGEM		S	0.399±0.008	CK1		–	0.077±0.003 S
PVYCP		S	0.404±0.007	CK2		–	0.081±0.004

[a] Data were average of four replicates. CK1, CK2: NC89 with no viral infection. *R* resistant *S* susceptible

apparent advantages. First, this method is not as time- and labor-consuming as others and provides a cost-effective way of producing of transgenic plants. Transgenic plants have increasingly raised concern for having potential negative ecological effects, such as heterologous encapsidation, complementation, synergy, and genetic flow between organisms. Second, this method is safer than pesticides, which potentially cause chemical residue

Table 6 ELISA detection of PVY in some tobacco plants interference with different PVY hpRNAs transcripts ($OD_{490\ nm}$)[a].

PVY hpRNA	No. of tobacco plant	Phenotype	Results of ELISA	PVY hpRNA	No. of tobacco plant	Phenotype	Results of ELISA
HC-Pro	3	R	0.098±0.001	NIb	7	R	0.087±0.009
	16	R	0.089±0.008		12	R	0.079±0.004
	42	R	0.088±0.003		36	R	0.082±0.006
	9	S	0.398±0.008		16	S	0.387±0.003
	25	S	0.396±0.001		22	S	0.372±0.004
	36	S	0.391±0.001		46	S	0.359±0.003
CP	9	R	0.090±0.007	PVY/pGEM		S	0.398±0.006
	17	R	0.087±0.002	TMVCP		S	0.408±0.005
	38	R	0.086±0.005	CK1		–	0.087±0.002
	14	S	0.390±0.003	CK2		–	0.392±0.005
	26	S	0.391±0.001				
	45	S	0.388±0.002				

[a] Data were average of four replicates. CK1, CK2: NC89 with no viral infection. *R* resistant, *S* susceptible

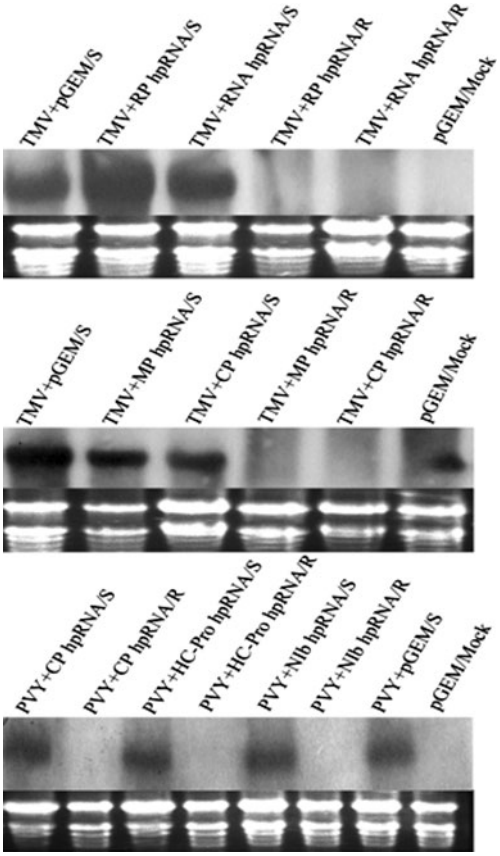

Fig. 4 Northern blot analysis of total RNA from tested tobacco plants. *R* local resistant, *S* susceptible. We used the TMV/pGEM or PVY/pGEM and pGEM/Mock of the same age as positive and negative controls. pGEM/Mock: the tested tobacco plants that only treated with the total RNA extracted from the pGEM-T plasmid. 18S and 25S rRNA were used to show that an equal amount of total RNA was loaded into each lane

accumulation in the environment. Third, dsRNAs produced by this method are stable in vivo for several days after inoculation, even after exposure of the plants to a rain storm [13, 30–32].

Since Tenllado and coworkers first showed that prokaryotic expression of dsRNA could protect plants from viral infection [13], many other researchers have conducted research in this area [30–32]. Zhang et al. [31] have studied the two genes responsible for protect plants against cucumber mosaic virus (CMV) infections. In previous research, we used the bacteriophage λ-dependent Red recombination system to knock out the rnc genes of *E. coli* strains and expressed the dsRNA of TMV CP gene [30]. At the present time, this type of antiviral research is confined to one or two functional genes of only a few viruses. In the present study, we selected seven important hot-spot sequences of TMV or PVY genome, the most major viruses of tobacco, and Northern blotting and local resistance analysis proved that the different hpRNAs could all prevent TMV or PVY infections in tobacco. However, the hpRNAs derived from hot-spot sequences of TMV or PVY genome had different resistance capacity. The resistance to TMV conferred by the hpRNA against the TMV MP was stronger than other TMV hpRNAs; the resistance to PVY conferred by the hpRNA

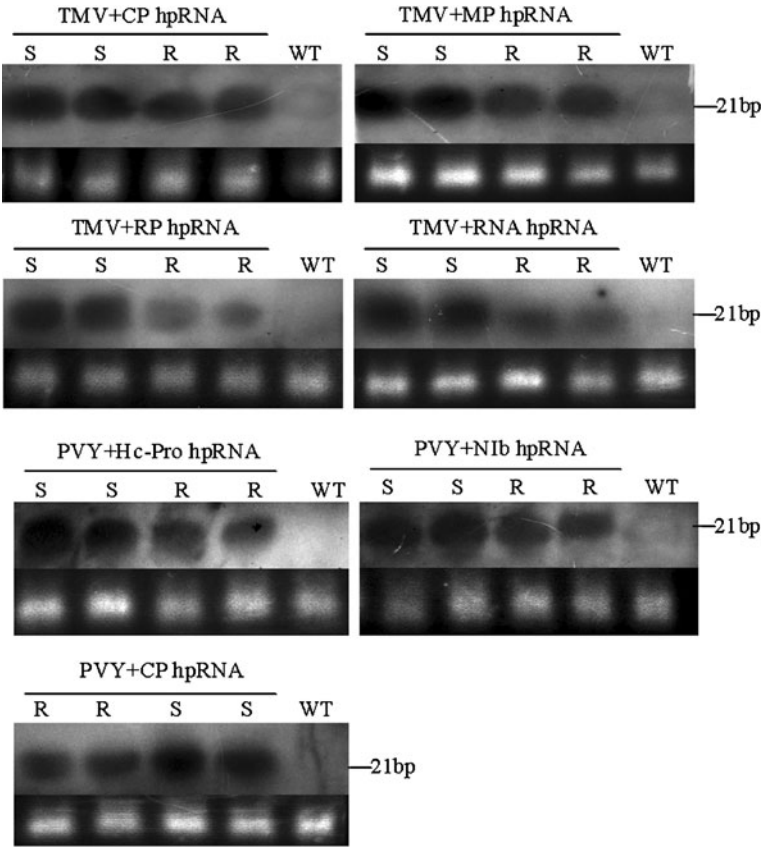

Fig. 5 Northern blot analysis of siRNAs from tested tobacco plants. *R* local resistant, *S* susceptible, *WT* wide-type tobacco. TMV or PVY RNA signals were detected in the susceptible tobacco but not in the resistant tobacco and pGEM/Mock tobacco. The size of all hybridized bands was about 21–25 nt, and 5S rRNA was used to show that an equal amount of small RNA fraction was loaded into each lane

against the NIb was better than that induced by any other PVY hpRNAs. The results of this study will provide helpful information to the effective selection of target gene sequences to cope with the viral infection in plants.

The double-stranded stem of an hpRNA determines the reaction specificity; it is the pivotal factor in inducing RNA silencing [33]. The length of the stem influences the capacity of the induced RNA silencing. In transgenic plant studies , the results of Wesley et al. [34] and Zhu et al. [28, 29] demonstrated that when the length of the stems was 200–800 bp, obvious silencing was elicited, with silencing efficiency as high as 60%–80%. The spacer of the hpRNAs had no direct relationship to RNA silencing, but some research showed that the length of the spacer could affect the transgenic posttranscriptional hpRNA formation and stability, thereby affecting the efficiency of gene silencing [35–39]. A short spacer is conducive to the formation of hpRNA, improving the stability of hpRNA, reducing the chances of attack by ribozyme, and inducing efficient gene silencing. On the other hand, when the lengths of the stems are too short, this can affect the efficiency of gene cloning, causing difficulties in vector construction [40, 41]. Li et al. [42] and Hirai et al. [43] demonstrated that a valid stem loop ratio was 1:1~4:1. Referring to the transgenic

proportion of the stem-spacer, we maintained the following proportions for this study: for TMV, we subcloned a 480-bp TMV MP gene as the stem of the hpRNA and subcloned a 120-bp GUS gene as the spacer region of the hpRNA; for PVY, we subcloned a 600-bp PVY CP gene as the stem of the hpRNA and subcloned a 120-bp GUS gene as the spacer region of the hpRNA. Local resistance results showed the highest resistance was as high as 71.68%, illustrating that it is feasible and effective for prokaryotic expression of dsRNA for antiviral research.

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