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Special origin of stem sequence influence the resistance of hairpin expressing plants against PVY

F. JIANG, B. WU, C. ZHANG, Y. SONG, H. AN, C. ZHU* and F. WEN*

State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong, 271018, P.R. China

Abstract

In this study, 16 hairpin RNA (hpRNA) vectors were constructed, each harboring 50 bp viral RNA sequence as the stem. They all targeted the coat protein (*CP*) gene of *Potato virus Y* (PVY). Virus resistance assay revealed that hairpin constructs targeting the anterior 200 bp regions of the *CP* gene were unable to induce virus resistance, while the 12 hpRNA constructs targeting posterior 600 bp regions induced high virus resistance up to 77.78 %. Northern blot analysis revealed that 50 bp-length hpRNA constructs could be transcribed efficiently and processed into siRNAs; however, no correlation between siRNA accumulation and degree of antiviral defense was observed. Results presented here indicated that the middle and 3′ end of the *CP* cDNA was important for hpRNA-mediated PVY resistance, improving the design of pathogen-derived hpRNA expression cassettes for transgenic plant against viruses.

Additional key words: *Agrobacterium tumefaciens*, hpRNA-mediated virus resistance, *Nicotiana tabacum*, RNA silencing.

Introduction

Potato virus Y (PVY), a member of genus *Potyvirus* from the family *Potyviridae*, is a main viral pathogen infecting economic crops such as potato and tobacco plants in many Asian and European countries. The viral genome is a single-stranded, positive-sense ribonucleic acid. Coding a polyprotein can be processed into 10 functional proteins, located in the N-to-C terminus: P1, HC-pro, P3, 6K1, CI, 6K2, NIa-Vpg, NIa-Pro, NIb, and CP (Urcuqui-Inchima *et al*. 2001).

To address the shortage of viral-resistant genes in plants, a number of strategies have been developed. Most of which are based on the concept of pathogen-derived resistance (Sanford and Johnson 1985, Kertbundit *et al.* 2007). Among these strategies, RNA-mediated virus resistance (RMVR), known as post-transcriptional gene silencing (PTGS) in plants, is considered a potential strategy with broad application value and practical significance in antivirus genetic engineering (Prins and Goldbach 1996, Mohanpuria *et al*. 2008).

Previous studies have shown that PTGS is a sequence-specific mRNA degradation mechanism (Zamore 2002, Almeida and Allshire 2005). In this process, double-stranded RNA (dsRNA) serves as the key trigger for inducing highly specific gene silencing. In plants, the hairpin RNA (hpRNA) precursor has been observed to be the most successful expression cassettes for expression of dsRNA (Wesley *et al*. 2001, Stoutjesdijk *et al*. 2002, Helliwell and Waterhouse 2003). Expression of virus-specific dsRNA mimics a viral infection, leading to the activation of a systemic virus-directed PTGS response in the host plant. These long dsRNAs are processed into siRNAs by a double strand-specific ribonuclease called Dicer (Bernstein *et al*. 2001). The generated siRNAs act as a guide for recognizing complementary invasive viral RNAs, allowing their degradation and conferring virus resistance. Therefore, the preexistence of a certain number of virus-specific siRNAs in plants could be a key determining factor for the efficient construction of a plant expression vector and acquired virus resistance (Abhary *et al*. 2006).

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Abbreviations: CaMV - cauliflower mosaic virus; CB - carbenicillin; CP - coat protein; dpi - day post-inoculation; dsRNA - double strand RNA; hpRNA - hairpinRNA; PTGS, - post-transcriptional gene silencing; PVY - *Potato virus Y*; SSC - sodium chloride/sodium citrate.

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* Corresponding authors; fax: (+86) 538 8226399, e-mail: zhchx@sdau.edu.cn

Different dsRNAs induce varying levels of RNA silencing activity (Yoshinari *et al*. 2004). Therefore, to achieve efficient virus resistance, it is important to screen appropriate target regions along the genome which confers high virus resistance. In previous research on PVY resistance, we observed that hpRNA constructs harboring 3' *CP* sequences were more efficient compared with those harboring 5' *CP* sequences at inducing PTGS (Li *et al*. 2007). To date, however, no systematic investigation of an entire gene has been conducted.

In this study, 16 hpRNA constructs targeting different regions of the *CP* gene of PVY are designed. It demonstrates that targeting different regions of an encode protein will engender various efficacies. The most effective hpRNA construct was derived from the middle and 3′ end of the *CP* gene.

Materials and methods

To design 16 small hpRNA constructs, the *CP* gene (GenBank GU550507) was divided into 16 regions. For every region, two PCR fragments were amplified from the whole length of the *CP* gene. Restriction enzyme cutting sites were added at each side of the fragments (Table 1). Two PCR fragments were invertly inserted into binary vector pROKII (Fig. 1). The 16 plant expression vectors obtained were dubbed pROK-CPs (pROK-CP1, pROK-CP2, pROK-CP3, pROK-CP16).

Constructed binary vectors pROK-CP, pROK-CPs, and pROKII (control) were individually transferred into *Agrobacterium tumefaciens* strain EHA105 (Bhattacharjee *et al*. 2010). The bacterial cultures and pressure infiltration were performed according to the method devised by Liu *et al.* (2003). For coinfiltration, the final absorbance ratio of the hpRNA-expressing strain to the full length CP-expressing strain pROK-CP was 3:1.

Nicotiana tabacum L. cv. NC89 plants were transformed with pROK-CPs or pROKII using the *Agrobacterium* LBA4404-mediated leaf disc method (Horsch *et al*. 1985). Transformants were selected in a Murashige and Skoog (MS) medium containing 250 mg dm^{-3} carbenicillin (CB) and 100 mg dm^{-3} kanamycin sulfate. Only a single transformant was selected from one callus and subsequently screened by PCR to detect the presence of respective transgenes. Through self-pollination, their seeds were selected in a germination medium containing kanamycin sulfate. These T_1 plants were used in subsequent virus resistance tests.

The virus inoculum was prepared by grinding PVY^N-infected leaves and diluting these in phosphate-buffered saline buffer at the ratio of 1:10 (m/v). Homogenate was employed to inoculate fully expanded upper leaf of T_1 progeny plants at the five- to six-true leaf stages (Burundukova *et al.* 2009). Resistant and susceptible plants were divided by observation of symptoms.

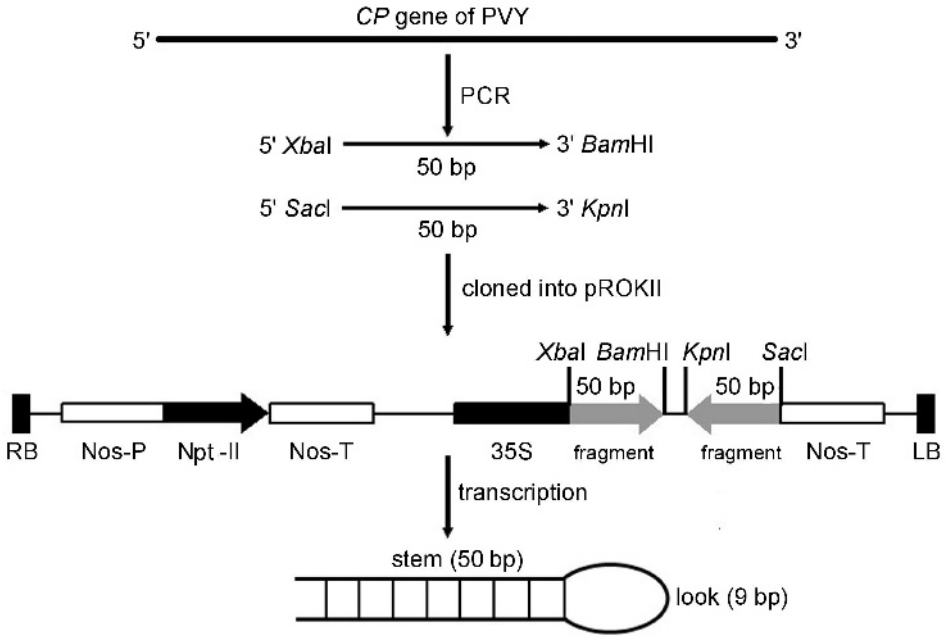

Fig. 1. Strategy for constructing hpRNA constructs. HpRNA constructs were cloned behind the CaMV 35S promoter in binary vector pROKII; RB - right border, LB - left border, Nos-P - promoter of the nopaline synthase gene, Npt-II - kanamycin resistance gene, Nos-T - terminator of the nopaline synthase gene.

Table 1. Description of oligonucleotide primers used in this study.

Fragment	Primer		Restriction enzyme
pROK-CP1-1	CP1-1-5′	5′ GC*TCTAGA*GCAAATGACACAATGCATG 3′	*Xba*I
	CP1-1-3′	5′ GC*GGATCC*GGTTTTGCATCTTTCTTGT 3′	*Bam*HI
pROK-CP1-2	CP1-2-5′	5′ GC*GAGCTC*GCAAATGACACAATGCATG 3′	*Sac*I
	CP1-2-3′	5′ GC*GGTACC*GGTTTTGCATCTTTCTTGT 3′	*Kpn*I
pROK-CP2-1	CP2-1-5′	5′ GC*TCTAGA*AGAGCAAGGCAGCATCCA 3′	*Xba*I
	CP2-1-3′	5′ GC*GGATCC*CATCCTTATCTTTTCCTTTG 3′	*Bam*HI
pROK-CP2-2	CP2-2-5′	5′ GC*GAGCTC*AGAGCAAGGCAGCATCCA 3′	*Sac*I
	CP2-2-3′	5′ GC*GGTACC*CATCCTTATCTTTTCCTTTG 3′	*Kpn*I
pROK-CP3-1	CP3-1-5′	5′ GC*TCTAGA*TGAATGCTGGTACATCTGG 3′	*Xba*I
	CP3-1-3′	5′ GC*GGATCC*GATAGCTTTGATTCTCGGC 3′	*Bam*HI
pROK-CP3-2	CP3-2-5′	5′ GC*GAGCTC*TGAATGCTGGTACATCTGG 3′	*Sac*I
	CP3-2-3′	5′ GC*GGTACC*GATAGCTTTGATTCTCGGC 3′	*Kpn*I
pROK-CP4-1	CP4-1-5′	5′ GC*TCTAGA*ACGCCCAAAATGAGAATGC 3′	*Xba*I
	CP4-1-3′	5′ GC*GGATCC*AAATTTAGCACGGTTGCTCC 3′	*Bam*HI
pROK-CP4-2	CP4-2-5′	5′ GC*GAGCTC*ACGCCCAAAATGAGAATGC 3′	*Sac*I
	CP4-2-3′	5′ GC*GGTACC*AAATTTAGCACGGTTGCTCC 3′	*Kpn*I
pROK-CP5-1	CP5-1-5′	5′ GC*TCTAGA*AGAACACTTGCTCGAGTAT 3′	*Xba*I
	CP5-1-3′	5′ GC*GGATCC*CCGAGTATTTGAAATATCAA 3′	*Bam*HI
pROK-CP5-2	CP5-2-5′	5′ GC*GAGCTC*AGAACACTTGCTCGAGTAT 3′	*Sac*I
	CP5-2-3′	5′ GC*GGTACC*CCGAGTATTTGAAATATCAA 3′	*Kpn*I
pROK-CP6-1	CP6-1-5′	5′ GC*TCTAGA*GGGCAACTCAATCACAGT 3′	*Xba*I
	CP6-1-3′	5′ GC*GGATCC*TGCCATCCGCACTGCTTC 3′	*Bam*HI
pROK-CP6-2	CP6-2-5′	5′ GC*GAGCTC*GGGCAACTCAATCACAGT 3′	*Sac*I
	CP6-2-3′	5′ GC*GGTACC*TGCCATCCGCACTGCTTC 3′	*Kpn*I
pROK-CP7-1	CP7-1-5′	5′ GC*TCTAGA*TACGACATAGGAGAAACTG 3′	*Xba*I
	CP7-1-3′	5′ GC*GGATCC*ACCATAAGCCCATTCATCA 3′	*Bam*HI
pROK-CP7-2	CP7-2-5′	5′ GC*GAGCTC*TACGACATAGGAGAAACTG 3′	*Sac*I
	CP7-2-3′	5′ GC*GGTACC*ACCATAAGCCCATTCATCA 3′	*Kpn*I
pROK-CP8-1	CP8-1-5′	5′ GC*TCTAGA*TTGGTGCATCGAAAATGGA 3′	*Xba*I
	CP8-1-3′	5′ GC*GGATCC*TAACCCAAACTCCGTTGAC 3′	*Bam*HI
pROK-CP8-2	CP8-2-5′	5′ GC*GAGCTC*TTGGTGCATCGAAAATGGA 3′	*Sac*I
	CP8-2-3′	5′ GC*GGTACC*TAACCCAAACTCCGTTGAC 3′	*Kpn*I
pROK-CP9-1	CP9-1-5′	5′ GC*TCTAGA*GATGGATGGGAACGAACAA 3′	*Xba*I
	CP9-1-3′	5′ GC*GGATCC*TCTCAACGATTGGTTTCAAC 3′	*Bam*HI
pROK-CP9-2	CP9-2-5′	5′ GC*GAGCTC*GATGGATGGGAACGAACAA 3′	*Sac*I
	CP9-2-3′	5′ GC*GGTACC*TCTCAACGATTGGTTTCAAC 3′	*Kpn*I
pROK-CP10-1	CP10-1-5′	5′ GC*TCTAGA*ATGCAAAACCAACCCTTAGG 3′	*Xba*I
	CP10-1-3′	5′ GC*GGATCC*TGCAACATCTGAGAAATGTG 3′	*Bam*HI
pROK-CP10-2	CP10-2-5′	5′ GC*GAGCTC*ATGCAAAACCAACCCTTAGG 3′	*Sac*I
	CP10-2-3′	5′ GC*GGTACC*TGCAACATCTGAGAAATGTG 3′	*Kpn*I
pROK-CP11-1	CP11-1-5′	5′ GC*TCTAGA*GAAGCGTATATAGAAATGCG 3′	*Xba*I
	CP11-1-3′	5′ GC*GGATCC*TATCGTGGCATATATGGTTC 3′	*Bam*HI
pROK-CP11-2	CP11-2-5′	5′ GC*GAGCTC*GAAGCGTATATAGAAATGCG 3′	*Sac*I
	CP11-2-3′	5′ GC*GGTACC*TATCGTGGCATATATGGTTC 3′	*Kpn*I
pROK-CP12-1	CP12-1-5′	5′ GC*TCTAGA*TGGTTTAATTCGAAATCTGC 3′	*Xba*I
	CP12-1-3′	5′ GC*GGATCC*CAAAGGCAAAGCGCGCTA 3′	*Bam*HI
pROK-CP12-2	CP12-2-5′	5′ GC*GAGCTC*TGGTTTAATTCGAAATCTGC 3′	*Sac*I
	CP12-2-3′	5′ GC*GGTACC*CAAAGGCAAAGCGCGCTA 3′	*Kpn*I
pROK-CP13-1	CP13-1-5′	5′ GC*TCTAGA*ACTTTTATGAGGTCACATCA 3′	*Xba*I
	CP13-1-3′	5′ GC*GGATCC*GTGCGCTTCCCTAGCCCT 3′	*Bam*HI
pROK-CP13-2	CP13-2-5′	5′ GC*GAGCTC*ACTTTTATGAGGTCACATCA 3′	*Sac*I
	CP13-2-3′	5′ GC*GGTACC*GTGCGCTTCCCTAGCCCCT 3′	*Kpn*I
pROK-CP14-1	CP14-1-5′	5′ GC*TCTAGA*ATTCAAATGAAGGCCGCAG 3′	*Xba*I
	CP14-1-3′	5′ GC*GGATCC*CCGAAAAGTCGAGGTTGA 3′	*Bam*HI
pROK-CP14-2	CP14-2-5′	5′ GC*GAGCTC*ATTCAAATGAAGGCCGCAG 3′	*Sac*I
	CP14-2-3′	5′ GC*GGTACC*CCGAAAAGTCGAGGTTGA 3′	*Kpn*I
pROK-CP15-1	CP15-1-5′	5′ GC*TCTAGA*GTTGGACGGTGGCATCAG 3′	*Xba*I
	CP15-1-3′	5′ GC*GGATCC*TGGTGTGCCTCTCTGTGT 3′	*Bam*HI

pROK-CP15-2	CP15-2-5′	5′ GC*GAGCTC*GTTGGACGGTGGCATCAG 3′	*Sac*I
	CP15-2-3′	5′ GC*GGTACC*TGGTGTGCCTCTCTGTGT 3′	*Kpn*I
pROK-CP16-1	CP16-1-5′	5′ GC*TCTAGA*CCGAGGATGTCTCTCCAA 3′	*Xba*I
	CP16-1-3′	5′ GC*GGATCC*CATGTTCTTGACTCCAAGT 3′	*Bam*HI
pROK-CP16-2	CP16-2-5′	5′ GC*GAGCTC*CCGAGGATGTCTCTCCAA 3′	*Sac*I
	CP16-2-3′	5′ GC*GGTACC*CATGTTCTTGACTCCAAGT 3′	*Kpn*I

Total RNA was extracted from tobacco leaves using *Trizol* reagent (*Invitrogen*, USA). Small RNAs were enriched through the removal of high molecular mass RNAs, using the method described by Tomita *et al*. (2004).

For Northern blot analysis**,** digoxigenin-labeled RNA probes for 1/4 length *NIb* gene (GenBank FJ560596) and respective 50 bp antisense fragment of the *CP* gene in hairpin construct were prepared following the manufacturer's instructions. For each sample, 20 μg of total RNA was electrophoresed in 1 % agarose gel containing 1 % formaldehyde and transferred onto a *Hybond-N*$^+$ membrane (*Amersham*, UK) with 20× SSC (Sambrook *et al.* 2001). For siRNA, digoxigenin-labeled riboprobes were prepared for the *CP* gene and its respective 50 bp fragments. A total of 30 μg of small RNA was separated in 15 % polyacrylamide gel containing 7 M urea; it was electro-transferred onto *Hybond-N*$^+$ membranes fixed by UV cross-linking for 1 min. Hybridization and chemiluminescent detection were performed following the manufacturer's instructions (*DIG Northern* starter kit, *Roche*, Germany).

Results

To examine whether the hpRNA constructs harboring 50 bp of the PVY-CP could activate the host RNA silencing machinery efficiently, expression levels of siRNA were detected. Small molecular RNAs were extracted from *N. benthamiana* leaves after transient infiltration by the *Agrobacterium* strains that harbored pROK-CPs or pROKII at 3 dpi. Northern blot analysis revealed siRNAs which were detected in all pROK-CPs-infiltrated leaves but not in control leaves (Fig. 2). Further, to investigate whether the siRNAs can down-regulate the expression of target RNA, the pROK-CP vector which expresses the full-length CP target gene was co-infiltrated into the *N. benthamiana* leaves with the pROK-CPs through agroinfiltration. Accumulation of the target transcripts was detected at 3 dpi. As expected, all 16 constructs exhibited decreased *CP* expression in the infiltrated regions compared with the control vector. This indicated that the said hpRNA constructs were recognized effectively and diced into biologically active siRNAs that could effectively cleave target RNA.

The 16 hpRNA constructs were introduced into *Nicotiana tabacum L.* var. NC89. Approximately 60 transgenic lines exhibiting kanamycin resistance and positive results in the PCR tests for each vector were regenerated. No abnormal developmental phenotypes were observed. The transgenic plants were challenged in three independent experiments using PVY^N. The 16 transgenic tobacco groups exhibited varying degrees of virus resistance (Table 2). The highest percentage of resistant plants (77.78 %) was obtained in the pROK-CP15 transgenic tobacco group. In groups pROK-CP7, pROK-CP8, pROK-CP9, pROK-CP10, pROK-CP11, pROK-CP14 and pROK-CP16, resistant plant ratios were approximately 60 %. Meanwhile, resistant plant ratios of

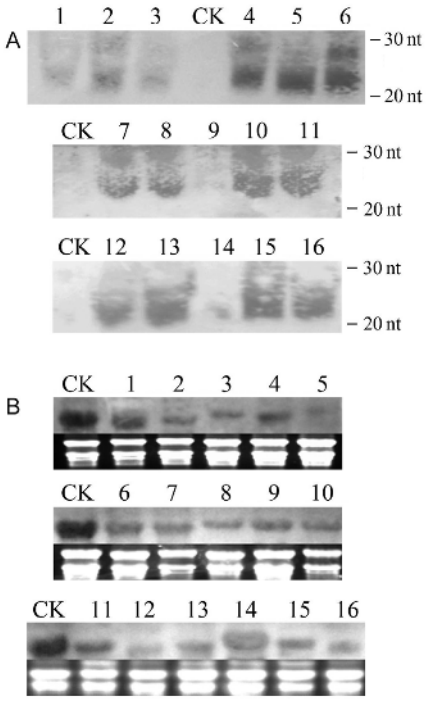

Fig. 2. Detection of PVY-CP-specific siRNAs and the reduction of PVY-CP target RNAs in transient expression assay. *A* - Detection of PVY-CP-specific siRNAs in transient expression assay. siRNAs hybridized with a DIG-labeled RNA probe for the full length CP gene. *B* - Detection of the reduction of PVY-CP target RNAs in transient expression assay. Co-infilitration pROK-CP with the pROK-CPs into the *N. benthamiana* leaves. The probe is same to *A*. *Lines* 1 - 16 represents respective constructs pROK-CP1, pROK-CP2, pROK-CP3…pROK-CP16, CK in each block represents pROKII. Loading controls were monitored of 28S rRNA or 5S rRNA.

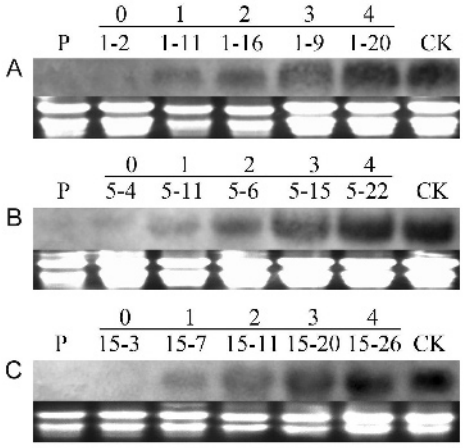

Fig. 3. Northern blot analysis of PVY RNAs accumulated in PVY-inoculated transgenic plants. A NIb-specific probe was used to detect the accumulation of PVY. *A*, *B* and *C* represent groups pROK-CP1, pROK-CP5, pROK-CP15. *Lanes* 0 - 4 showing the four-grade disease index: 0 - no symptoms, 1 - mild green-fading on one top leaf, 2 - mild vein-clearing symptom on more than two leaves, 3 - pronounced vein-clearing on older leaves which later developed into mosaic, 4 - pronounced vein-necrosis symptom and plants severely stunted. P - non-infecting transgenic plants; CK - PVY infecting transgenic plants containing pROKII.

groups pROK-CP5, pROK-CP6, pROK-CP12, and pROK-CP13 were approximately 10 - 20 %. None of the resistant plants were obtained from the pROK-CP1, pROK-CP2, pROK-CP3, and pROK-CP4 groups. These results indicated that a large number of hpRNA constructs could confer virus resistance in transgenic plants; however, the hairpin constructs harboring different cDNA regions of the CP gene possessed different silencing efficiencies.

To examine whether the observed virus resistance correlated with a reduction in viral RNA replication, three transgenic groups were selected, pROK-CP1, pROK-CP5, and pROK-CP15. These groups substituted no virus resistance, imperfectly enhanced resistance, and highly enhanced resistance, respectively. Five plants were selected from each group, and each inoculation plant exhibited different disease index grades at 14 dpi. Northern blot analysis with a *NIb*-specific probe revealed that susceptible plants (disease index grade of 4) accumulated approximately equal amounts of viral RNA compared with the control type. Accumulation levels of PVY RNA in plants with disease index grade of 1, 2 decreased compared with the susceptible type. In plants that possessed a disease index grade of 0, PVY genomic RNAs were undetected (Fig. 3). The result revealed the existence of a negative relationship between resistance and the amount of PVY RNA accumulation in the transgenic plant.

Furthermore, correlation between resistance and the accumulation of hpRNA construct transcripts was analyzed. Susceptible transgenic plants accumulated approximately equal amounts of transcripts compared with control type, while all resistant transgenic plants exhibited a reduction in mRNA level (Fig. 4). This suggested that the hpRNA constructs could be transcribed efficiently in the plant, and that the resistance was correlated with a reduction in transgenic RNA accumulation.

Table 2. Resistance analysis of PVY^N *CP* gene derived transgenic NC89 plants challenged with PVY^N. CK - control (vector-transformed) plants.

Transgenic group	Number of plants inoculated	Number of resistant plants	Ratio [%]
pROK-CP1	60	0	0
pROK-CP2	55	0	0
pROK-CP3	70	0	0
pROK-CP4	66	0	0
pROK-CP5	66	18	27.15±2.09
pROK-CP6	48	3	6.27±0.39
pROK-CP7	68	46	67.65±2.22
pROK-CP8	72	51	70.83±4.17
pROK-CP9	50	33	66.01±1.13
pROK-CP10	62	38	61.34±1.63
pROK-CP11	56	37	66.04±2.07
pROK-CP12	55	13	23.59±2.36
pROK-CP13	45	5	11.11±3.85
pROK-CP14	63	35	55.56±1.39
pROK-CP15	72	56	77.78±2.41
pROK-CP16	55	37	67.25±1.01
CK	40	0	0

Table 3. Comparison of efficiency of gene silencing with the H-b index and ΔG. The H-b index was determined according to Luo and Chang (2004); the ΔG of structures were calculated using the web (http://www.bioinfo.rpi.edu/applications/mfold).

Target sequence	ΔG [kJ mol^{-1}]	H-b index
CP1	-201.77	32.82
CP2	-99.21	71.50
CP3	-92.09	70.5
CP4	-152.79	68.13
CP5	-79.95	55.63
CP6	-127.67	83.00
CP7	-135.63	74.13
CP8	-121.39	66.00
CP9	-69.49	79.19
CP10	-121.81	83.38
CP11	-57.77	59.13
CP12	-147.77	84.88
CP13	-89.16	78.63
CP14	-83.72	70.88
CP15	-87.07	59.81
CP16	-162.42	70.88

To determine if the resistance was correlated with the *in vivo* level of expressed siRNAs, siRNA was extracted from resistant and susceptible plants of each transgenic group prior to PVY inoculation. Northern blot analysis revealed that siRNAs were present in all selected resistant and susceptible transgenic plants; in contrast, these were rarely detected in the wild type plants (Fig. 5). No obvious correlation was observed between the expression level of sequence-specific siRNAs and virus resistance. This result suggested that the accumulation level of siRNAs may not be the sole determinant of susceptibility to PVY infection.

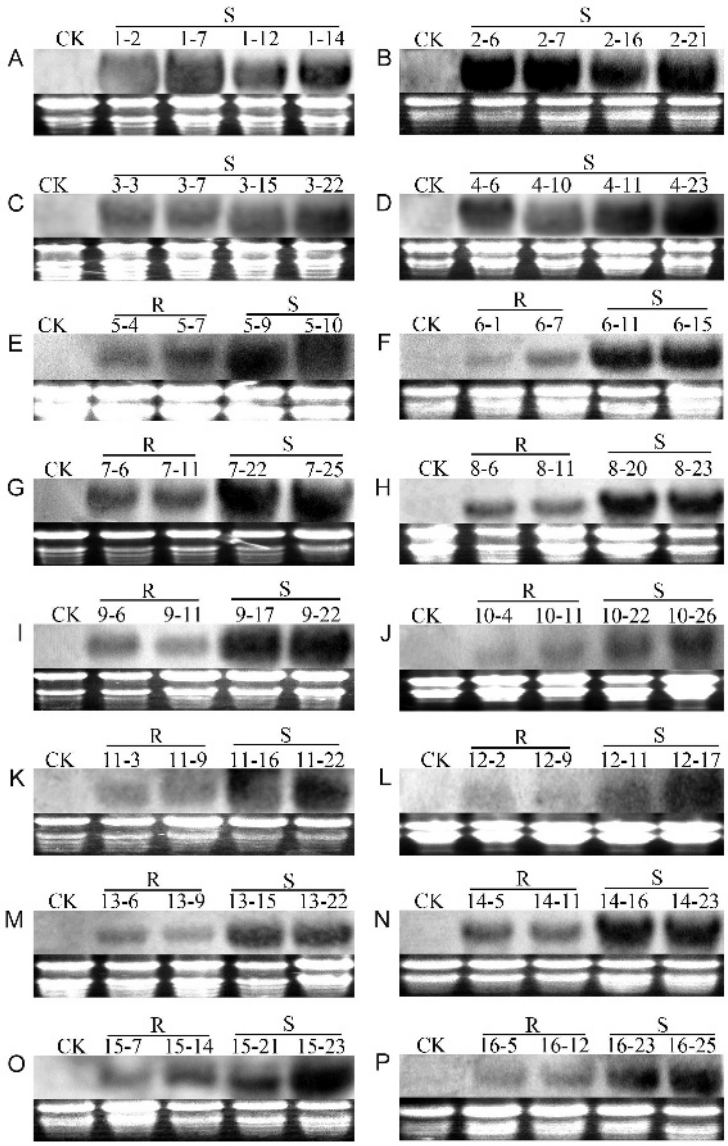

Fig. 4. Northern blot analysis of the transcripts in transgenic plants (*A* - *P*). Each block represents an individual hybridization with an individual RNA probe, respective 50 bp fragment of the antisense fragment in hairpin construct. rRNA was used to show that an equal amount of total RNA was loaded (*lower part*). CK - transgenic plants containing pROKII, S - susceptible plant, R - resistant plant.

Discussion

Post-transcriptional gene silencing mediated by pathogen-derived hairpin RNA has become a powerful tool for conferring virus resistance in transgenic plants. At present, a gene or a long sequence of the virus (> 300 nt) is commonly utilized in research (Smith *et al*. 2000, Missiou *et al*. 2004). A long viral cDNA fragment may offer higher gene silencing efficiency, but result in more off-target effect or virus recombinant. It will influence the

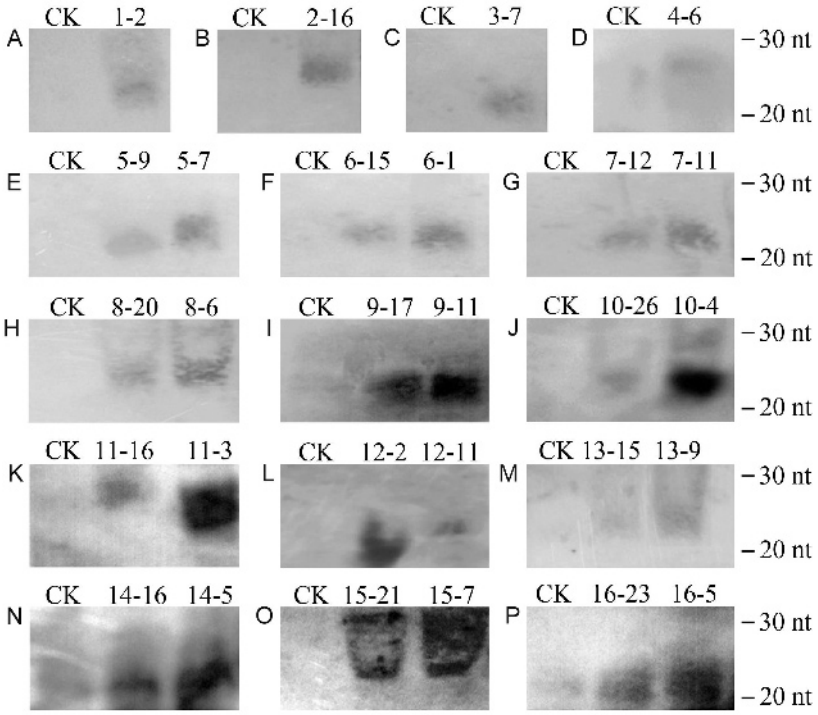

Fig. 5. Northern blot analysis of siRNA from transgenic plants. Each block represents an individual hybridization with an individual RNA probe, transcribed from the individual region of CP gene corresponding to the stem sequence. CK - transgenic plants containing pROKII.

endogenous gene expression of the plant or trigger environmental biosafety problems. On the other hand, short hairpin RNA (shRNA, 19 - 29 nt) can generate just one type of specific siRNA, resulting in less efficiency. In addition, not all siRNAs result in equal efficiency, thereby the choice of shRNA is a puzzle for the investigator (Holen *et al.* 2002, Luo and Chang 2004). In our work, 50 bp dsRNA is sufficient for PTGS, making it the ideal choice for the target sequence. In addition, it also enhances biosafety and efficiency of the silencing construct.

Meanwhile, RNA-mediated virus resistance is a sequence-dependent RNA degradation pathway. However, resistance analysis revealed that not all hpRNA constructs targeting different regions of PVY CP gene were equally effective. This phenomenon is consistent with other investigations (Vickers *et al*. 2003, Duan *et al*. 2008). Several hypotheses have been proposed in an attempt to explain this phenomenon. Among these hypotheses, local RNA target secondary structure and accessibility factor may be the most convincing (Nykänen *et al*. 2001, Holen *et al*. 2002, Overhoff *et al*. 2005). In this study, the structure of the target mRNA was analyzed, and it was observed that there was indeed a correlation between virus resistance and the accessibility of the target site represented by the ΔG_{loc} and H-b index (Luo and Chang 2004, Schubert *et al*. 2005). Regions with the lowest H-b index (< 70), pROK-CP8, pROK-CP11 and pROK-CP15, exhibited high virus resistance at 70.83, 66.04 and 77.78 %, respectively (Table 3). For the ΔG_{loc}, the regions whose absolute values of $\Delta G_{loc,}$ were the lowest displayed the highest virus resistance. For a thorough examination of the relationship between silencing efficiency and targeted mRNA, further work is necessary.

It is interesting to note that in our research, hairpin constructs harboring anterior 200 bp regions of the *CP* gene were unable to induce virus resistance. Meanwhile, siRNAs were detected and the expression of the *CP* gene was down-regulated in transient assay. It is speculated that this could be influenced by the local protein which exists during viral replication, while certain proteins are not produced during the transient assay (Holen *et al.* 2002). Therefore, although *Agrobacterium*-mediated transient expression assay is a useful screening technique for detecting silencing efficiency, it remains a surrogate for hpRNA constructs in transgenic plants.

Otherwise, silencing effect has been measured at the RNA level. It was observed that the accumulation of specific siRNAs in resistant plants was no greater than that in susceptible plants. One possible explanation is that the effective siRNAs, which are expected to be active and target-accessible, may not always be equally generated in transgenic plants despite similarities in total siRNA level.

In conclusion, a systematic analysis was conducted to examine the virus resistance conferred by 16 hpRNA constructs harboring different regions of the PVY-CP gene. The results demonstrated that the levels of virus

resistance varied between hpRNAs targeting different regions of the gene, and that the middle and 3′ end of the CP cDNA induced more effective gene silencing. This work is envisioned to facilitate future engineering of PVY-CP to confer virus resistance, and provide guidance on hpRNA-mediated anti-virus assay.

References

Abhary, M.K., Anfoka, G.H., Nakhla, M.K., Maxwell, D.P.: Post-transcriptional gene silencing in controlling viruses of the tomato yellow leaf curl virus complex. - Arch. Virol. **151**: 2349-2363, 2006.

Almeida, R., Allshire, R.C.: RNA silencing and genome regulation. - Trends Cell Biol. **15**: 251-258, 2005.

Bernstein, E., Caudy, A.A., Hammond, S.M., Hannon, G.J.: Role for a bidentate ribonuclease in the initiation step of RNA interference. - Nature **409**: 363-366, 2001.

Bhattacharjee, B., Mohan, M., Nair, S.: Transformation of chickpea: effect of genotype, explant, *Agrobacterium*-strain and composition of culture medium. - Biol. Plant. **54**: 21-32, 2010.

Burundukova, O.L., Sapotsky, M.V., Kochetov, A.V., Trifonova, E.A., Malinovsky, V.I.: Dark and light green tissues of tobacco leaves systemically infected with tobacco mosaic virus. - Biol. Plant. **53**: 294-300, 2009.

Duan, C.G., Wang, C.H., Fang, R.X., Guo, H.S.: Artificial microRNAs highly accessible to targets confr efficient virus resistance in plants. - J. Virol. **82**: 11084-11095, 2008.

Helliwell, C., Waterhouse, P.: Constructs and methods for high-throughput gene silencing in plants. - Methods **30**: 289-295, 2003.

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., Prydz, H.: Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. - Nucl. Acids Res. **30**: 1757-1766, 2002.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholz, D., Rogers, S.G., Fraley, R.T.: A simple and general method for transforming genes into plants. - Science **227**: 1229-1231, 1985.

Kertbundit, S., Pongtanom, N., Ruanjan, P., Chantasingh, D., Tanwanchai, A., Panyim, S., Juricek, M.: Resistance of transgenic papaya plants to papaya ringspot virus. - Biol. Plant. **51**: 333-339, 2007.

Li, P., Song, Y.Z., Liu, X.L., Zhu, C.X., Wen, F.J.: Study of virus resistance mediated by inverted repeats derived from 5′ and 3′ ends of coat protein gene of *Potato virus Y*. - Acta phytopathol. sin. **37**: 69-72, 2007.

Liu, Z.Z., Wang, J.L., Huang, X., Xu, W.H., Liu, Z.M., Fang, R.X.: The promoter of a rice glycine-rich protein gene, Osgrp-2, confers vascular-specific expression in transgenic plants. - Planta **216**: 824-833, 2003.

Luo, K.Q., Chang, D.C.: The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. - Biochem. Biophys. Res. Commun. **318**: 303-310, 2004.

Missiou, A., Kalantidis, K., Boutla, A., Tzortzakaki, S., Tabler, M., Tsagris, M.: Generation of transgenic potato plants highly resistant to potato virus Y (PVY) through RNA silencing. - Mol. Breed **14**: 185-197, 2004.

Mohanpuria, P., Rana, N.K., Yadav, S.K.: Transient RNAi based gene silencing of glutathione synthetase reduces glutathione content in *Camellia sinensis* (L.) O. Kuntze somatic embryos. - Biol. Plant. **52**: 381-384, 2008.

Nykänen, A., Haley, B., Zamore, P.D.: ATP requirements and small interfering RNA structure in the RNA interference pathway. - Cell **107**: 309-321, 2001.

Overhoff, M., Alken, M., Far, R.K., Lemaitre, M., Lebleu, B., Sczakiel, G., Robbins, I.: Local RNA target structure influences siRNA efficacy: a systematic global analysis. - J. mol. Biol. **348**: 871-881, 2005.

Prins, M., Goldbach, R.: RNA-mediated virus resistance in transgenic plants. - Arch. Virol. **141**: 2259-2276, 1996.

Sambrook, J., Russell, D.W.: Molecular Cloning: a Laboratory Manual. - Cold Spring Harbour Laboratory Press, New York 2001.

Sanford, J.C., Johnson, S.A.: The concept of parasite-derived resistance: deriving resistance genes from the parasite own genome. - J. theor. Biol. **115**: 395-405, 1985.

Schubert, S., Grünweller, A., Erdmann, V.A., Kurreck, J.: Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. - J. mol. Biol. **348**: 883-893, 2005.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., Waterhouse, P.M.: Gene expression: total silencing by intron-spliced hairpin RNAs. - Nature **407**: 319-320, 2000.

Stoutjesdijk, P.A., Singh, S.P., Liu, Q., Hurlstone, C.J., Waterhouse, P.A., Green, A.G.: Hp-RNA-mediated targeting of the *Arabidopsis FAD2* gene gives highly efficient and stable silencing. - Plant Physiol. **129**: 1723-1731, 2002.

Tomita, R., Hamada, T., Horiguchi, G., Iba, K., Kodama, H.: Transgene overexpression with cognate small interfering RNA in tobacco. - FEBS Lett. **573**: 117-120, 2004.

Urcuqui-Inchima, S., Haenni, A.L., Bernardi, F.: Potyvirus proteins: a wealth of functions. - Virus Res. **74**: 157-175, 2001.

Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M., Baker, B.F.: Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. - J. biol. Chem. **278**: 7108-7118, 2003.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G., Waterhouse, P.M.: Construct design for efficient, effective and high throughput gene silencing in plants. - Plant J. **27**: 581-590, 2001.

Yoshinari, K., Miyagishi, M., Taira, K.: Effects on RNAi of the tight structure, sequence and position of the targeted region. - Nucl. Acids Res. **32**: 691-699, 2004.

Zamore, P.D.: Ancient pathways programmed by small RNAs. - Science **296**: 1265-1269, 2002.