DOI: 10.1007/s10535-013-0314-4 BIOLOGIA PLANTARUM 57 (3): 547-554, 2013

Viral resistance mediated by shRNA depends on the sequence similarity and mismatched sites between the target sequence and siRNA

L. ZHANG, X. XIE, Y. SONG, F. JIANG, C. ZHU*, and F. WEN

State Key Laboratory of Crop Biology, Shandong Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, Shandong, 271018, P.R. China

Abstract

Viral resistance can be effectively induced in transgenic plants through their silencing machinery. Thus, we designed nine short hairpin RNAs (shRNA) constructs to target nuclear inclusion protein b (*NIb*), helper component proteinase (*HC-Pro*), cylindrical inclusion protein (*CI*), and viral protein genome linked (*VPg*) genes of *Potato virus Y* (PVY^N) and *Tobacco etch virus* (TEV-SD1). The shRNAs were completely complementary to the genes of PVY^N, and contained 1-3 nt mismatches to the genes of TEV-SD1. To study the specificity of gene silencing in shRNA-mediated viral resistance, the constructs were introduced into tobacco plants. The results of viral resistance assay reveal that these nine kinds of transgenic tobacco plants could effectively induce viral resistance against both PVY^N and TEV-SD1, and the shRNA construct targeting the *NIb* gene showed higher silencing efficiency. Northern blot and short interfering RNA (siRNA) analyses demonstrated that the viral resistance could be attributed to the degradation of the target RNA through the RNA silencing system. Correlation analysis of siRNA sequence characteristics with its activity suggests that the secondary structure stability of the antisense strand did not influence siRNA activity; 1 to 3 nt 5' end of the sense strand caused a significant effect on siRNA activity where the first base, such as U, was favourable for silencing; the base mismatch between the siRNA and the target gene may be more tolerated in the 5' end.

Additional key words: *Nicotiana benthamiana*, *Potato virus Y*, RNA silencing, *Tobacco etch virus.*

Introduction

Post-transcriptional gene silencing or RNA interference (RNAi) is a natural defense mechanism that enables the plants to control viral infections by damaging the structure of invasive RNA (Lindbo *et al*. 1993). In transgenic plants, this resistance depends only on the transcription of virus-derived sequence and not on the expression of the corresponding protein (Cogoni *et al*. 1994). The double-stranded RNA (dsRNA), particularly the short interfering RNA (siRNA) that contains 21 to 22 nucleotide fragments with 2-nucleotide 3-overhangs, triggers the endogenous silencing mechanism in plants (Szittya *et al*. 2003). By now, there are mainly two methods to produce siRNA. One is to directly synthesize siRNA *in vitro*, whereas the other is to construct a dsRNA expression vector and transfer that into the plants to generate siRNA by the *in vivo* gene silencing mechanism. The harpin RNA (hpRNA) precursor has been demonstrated to be the most successful expression cassette to express dsRNA in plants (Wesley *et al*. 2001, Ramesh *et al*. 2007, Fahim *et al*. 2010).

In the field, several viral diseases often attack organisms simultaneously. Cultivating anti-multi-viral crops can significantly increase crop yields. In previous studies, the common method involves cloning different kinds of viral coat protein genes into different expression cassettes to obtain relatively broad resistance against the corresponding viral strains (Fitchen *et al*. 1993). However, the coat protein genes may also recombine with other heterogeneous viruses in nature and produce dangerous viruses that are transmittable and prevalent

Received 6 June 2012, *accepted* 7 November 2012.

Abbreviations: CaMV - cauliflower mosaic virus; CI - cylindrical inclusion protein; dsRNA - double strand RNA; HC-Pro - helper component proteinase; hpRNA - hairpinRNA; Nib - nuclear inclusion protein b; PVY - *Potato virus Y*; shRNA - short hairpin RNA; SSC - sodium chloride/sodium citrate; VPg - viral protein genome linked.

Acknowledgments: This work was partially supported by the National Natural Science Foundation of China (No. 31272113) and the National Natural Science Foundation of Shandong Province (ZR2012CM001).

* Corresponding author; fax: (+86) 538 8241245, e-mail: zhchx@sdau.edu.cn

(Bucher *et al*. 2006). As there is no transgenic mRNA accumulation in transgenic plants, RNAi-mediated virus resistance has the advantage of biosafety (Lindbo *et al*. 1992). Based on the RNAi, two main approaches in cultivating anti-multi-viral transgenic plants are used. One approach is to construct a chimeic hpRNA derived from different viral gene fragments (cDNA; Bucher *et al*. 2006, Yan *et al*. 2007, Zhu *et al*. 2009). The other approach is to express one conservative sequence among different viruses (viral strains; Lucioli *et al*. 2003, Xu *et al*. 2009).

Potyviruses (genus *Potyvirus*, family *Potyviridae*) are the largest family of plant-infecting viruses. They can infect many kinds of plants including *Solanaceae*, *Chenopodiaceae*, *Leguminosae*, *Cucurbitaceae*, and so on. *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV) are two typical species of Potyviruses. In this study, we cultivated a resistant plant against PVY^N and TEV using a single short hairpin RNAs (shRNA) construct. The result shows that no positive correlation between virus resistance and target sequence similarity was observed and that different bases of the sense strand contributed differently to the identification of the target sequence.

Materials and methods

Potato virus Y (PVY^N; GenBank No. EU182576) and *Tobacco etch virus* (TEV-SD1; GenBank No. EF470242) were selected as the target viruses. The highly conserved shRNA target sequences in the genome were confirmed by comparing PVY^N with TEV-SD1 using *DNAman 5.2.2* software. Then, the shRNA) target site was identified using the *siRNA Target Finder* program (http:/www.ambion.com/techlib/misc/siRNA_finder.html). Based on the selected nucleotide sequences of shRNA, two single-strand DNAs were directly synthesized and annealed to form a double-strand DNA (dsDNA). Then, these dsDNAs (denoted S1, S2, S3, S4, S5, S6, S7, S8, and S9) with overhangs of the *Bam*HI and *Kpn*I sites were ligated into the corresponding restriction enzyme sites of the binary vector pROK II (maintained in the laboratory of Shandong Agricultural University, Key Laboratory of Crop Biology) to construct recombinant binary expression vectors (Fig. 1).

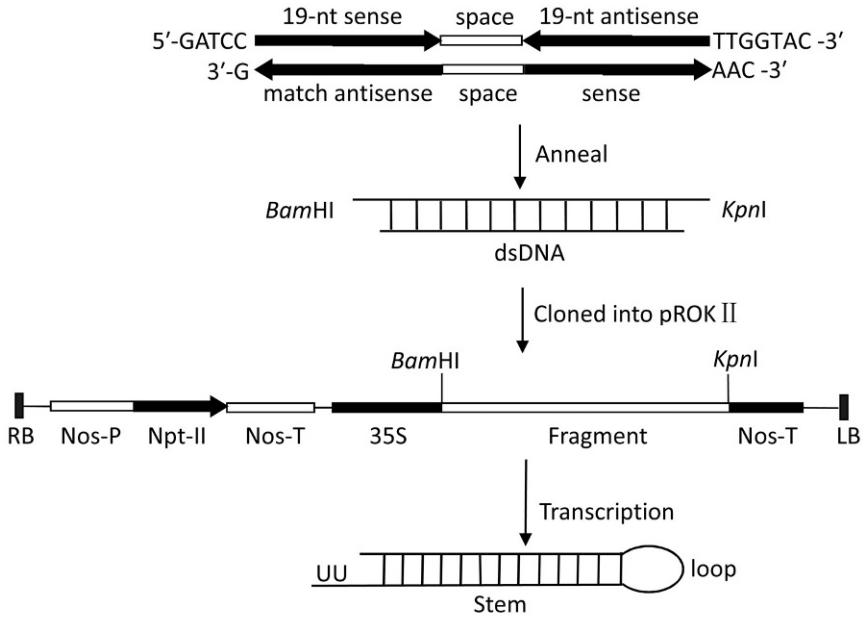

Fig. 1. The construction strategy of the binary vectors: RB - T-DNA right border, LB - T-DNA left border, Nos-P - nitrogen oxide systems (Nos) promoter, *npt* II - neomycin phosphotransferase gene, Nos-T - Nos terminator, 35s - cauliflower mosaic virus (CaMV) 35S promoter, Fragment - target cDNA.

For the efficiency detection of designed shRNA constructs, the nine recombinant binary vectors and pROK II (negative control) were separately transferred into *Agrobacterium tumefaciens* strain EHA105 (maintained in the laboratory of Shandong Agricultural University, Key Laboratory of Crop Biology). *Nicotiana benthamiana* Domin cv. NC89 plants were cultivated in a growth chamber under a 16-h photoperiod.

Ten six-week-old plants for each vector were injected through pressure infiltration. After 24 h, these plants were manually inoculated with PVY^N; the symptoms were observed and recorded every 4 d.

For viral resistance assay, the recombinant binary vectors and control vector pROK II were introduced into *A. tumefaciens* strain LBA4404 by freezing and thawing and introduced into tobacco NC89 through leaf disk transformation. Transformants were selected in a Murashige and Skoog (MS) medium containing 250 mg dm^{-3} carbenicillin (CB) and 100 mg dm^{-3} kanamycin sulfate, and further grown in a growth chamber at 25 °C, air humidity 65 of %, a 16-h photoperiod and irradiance of 300 - 400 μmol m^{-2} s^{-1}. The transgenic plants were verified using PCR to detect the presence of the respective transfer fragment. The viral inoculum was prepared by grinding the virus-infected leaves in phosphate buffer (pH 7.4) at a ratio of 1:10 (m/v). The transgenic plants were manually inoculated with PVY^N or TEV-SD1. Wild tobacco plants were used as the negative control. After a week, the symptoms were observed and recorded every 3 d. Virus infection in plants was detected by indirect enzyme-linked immunosorbent assay (ELISA) 20 d after inoculation (Guo *et al*. 2001). ELISA was carried out using a polyclonal antiserum to the PVY or TEV. Each line was divided into three groups for analysis.

Total RNA was extracted with *Trizol* reagent (*Invitrogen*, Carlsbad, USA) and dissolved in diethylpyrocarbonate-H_2O (DEPC-H_2O). The concentration of RNA was measured using electrophoresis and spectrophotometry. Total RNA (10 μg) was electrophoresed on a 1.2 % (m/v) agarose gel containing formaldehyde and transferred to *Hybond*^{TM}-N^+ membranes (*Amersham*, Chalfont, UK) with 20× SSC. Probe preparation and Northern blot hybridization were performed using *DIG* hybridization kit (*Roche*, Mannheim, Germany) according to the manufacturer's instructions.

The siRNAs were extracted using the *PureLink*^{TM} miRNA isolation kit (*Invitrogen*) according to the manufacturer's instructions. The samples were heat-treated in formamide buffer, loaded onto a 15 % (m/v) polyacrylamide gel containing 7 M urea, electro-transferred to a *Hybond*^{TM}-N^+ membrane using the trans-blot (*DYCP-40C*), and fixed by UV cross-linking. The subsequent hybridization used the same procedures as in total RNA Northern blot.

Results

Based on the comparison of the sequence similarity, we chose nine target sequences according to the native characteristics of siRNA (Donald *et al*. 2009). The target sequences were located in different regions (S1, S2, and S8 in *NIb* gene; S3, S4, and S7 in *HC-Pro* gene; S5 and S6 in *CI* gene; S9 in *VPg* gene) in the PVY^N genome which exhibited different sequence similarities to those in the TEV genome (85.7 % in S1, S2, S3, and S7; 90.5 % in S4, S5, and S6; 95.2 % in S8 and S9). Additionally, the target sequences with the same similarity revealed different mismatched bases; the mismatched sites were either located in the 3' end, 5' end, or in the middle regions of PVY^N and TEV-SD1 sequences (Table 1). The recombinant binary vectors including pR-NIB-S1,

Table 1. The siRNA target sequence with high similarity between TEV-SD1 and PVY^N and number and position of mismatched bases (SC - the sense chain; ASC - the antisense chain; the bases in *italic* are the mismatched bases).

No.		Sequence alignment	siRNA	Sequence alignment	Number and positions	Target genes
S1	TEV	AATTTGTACACTGAGAT*A*GT*G*AATTTGT	SC	UUUGUACACUGAGAUUAUU	3; 3' end	NIb
	PVY	ACACTGAGAT*TA*T*T*	ASC	AAUAAUCUCAGUGUACAAAUU		
S2	TEV	AAG*AC*AC*A*GTTGTGGTTTATGAAG*GA*A	SC	GGAAGAGUUGUGGUUUAUG	3; 5' end	NIb
	PVY	*G*AGTTGTGGTTTATG	ASC	CAUAAACCACAACUCUUCCUU		
S3	TEV	AA*T*TACGTGTATCC*AT*GTTGTAA*C*TACG	SC	CUACGUGUAUCCCAGUUGU	3; 3', 5' end	HC-Pro
	PVY	TGTATCC*CA*GTTGT	ASC	ACAACUGGGAUACACGUAGUU		
S4	TEV	AAAGCTTGGA*GC*G*T*GGCCAACAAAGCT	SC	AGCUUGGAACCUGGCCAAC	2; middle	HC-Pro
	PVY	TGGA*ACC*TGGCCAAC	ASC	GUUGGCCAGGUUCCAAGCUUU		
S5	TEV	AAC*T*GAGGG*G*CACTTCATGGAAAC*C*GA	SC	CCGAGGGACACUUCAUGGA	2; 5' end, middle	CI
	PVY	GGG*A*CACTTCATGGA	ASC	UCCAUGAAGUGUCCCUCGGUU		
S6	TEV	AAC*T*AACAT*C*ATTGAGAATGGAAC*C*AA	SC	CCAACAUAAUUGAGAAUGG	2; 5' end, middle	CI
	PVY	CAT*A*ATTGAGAATGG	ASC	CCAUUCUCAAUUAUGUUGGUU		
S7	TEV	A*G*GATGCAAAGGA*C*TTCAC*C*AA*A*GATG	SC	GAUGCAAAGGAUUUCACUA	3; 3', 5' end,	HC-Pro
	PVY	CAAAGGA*T*TTCAC*T*A	ASC	UAGUGAAAUCCUUUGCAUCUU	middle	
S8	TEV	AATGCTGCG*A*AATTTGTACACAATGCTG	SC	UGCUGCGCAAUUUGUACAC	1; middle	NIb
	PVY	CG*C*AATTTGTACAC	ASC	GUGUACAAAUUGCGCAGCAUU		
S9	TEV	AACATGTATGGGTT*T*GATCCAAACATGT	SC	CAUGUAUGGGUUCGAUCCA	1; middle	VPg
	PVY	ATGGGTT*C*GATCCA	ASC	UGGAUCGAACCCAUACAUGUU		

pR-NIB-S2, pR-HC-S3, pR-HC-S4, pR-CI-S5, pR-CI-S6, pR-HC-S7, pR-NIB-S8, and pR-VPG-S9 were constructed by directly annealing the synthesized single-stranded DNA (ssDNA.).

To examine whether the shRNA constructs could efficiently activate the host RNA silencing machinery, we measured the PVY^N resistance using the transient expression assay. No symptom was visible in the plants injected with recombinant binary vectors until the 12^{th} day. On the 16^{th} day, at least one *N. benthamiana* with no disease was present in the recombinant binary vector experimental group whereas all the plants injected with pROK II were infected indicating that the recombinant binary vectors could effectively inhibit the reproduction of PVY^N in transient expression. The most effective vector was the pR-NIB-S1 for which 3 out of the 10 infected plants remained healthy. In addition, we detected the expression of siRNA in the test plants. The hybridization results demonstrate that the recombinant shRNA vectors could be transcribed to generate specific siRNAs (Fig. 2).

To examine whether the designed shRNA constructs, that targeted two viral genomes with different sequence similarities, could induce viral resistance against both PVY^N and TEV, firstly we manually inoculated all the transgenic and wild tobacco NC89 plants with PVY^N. After two weeks, all the wild tobacco plants showed the typical symptom of PVY^N infection. In comparison, the plants transformed with the recombinant vectors were divided into two types, resistant and susceptible. The resistant plants were completely symptomless and no viral content was detected using ELISA (data not shown) whereas the susceptible plants were severely affected with distinct vein-clearing and mosaic symptoms (Table 2). These results show that the shRNA expression vectors (pR-NIB-S1, pR-NIB-S2, and pR-NIB-S8), which target the *NIb* gene and the pR-NIB-F4 construct targeting the *HC-Pro* gene, provided high resistance efficiency. It was also identified that siRNA activity might be affected by the different functional genes or by the location of the target mRNA within the full-length sequences (the *NIb* and *HC-Pro* genes were located in 5' end of viral genome). Secondly, the PVY^N-resistant plants were produced through asexual propagation and inoculated with PVY^N or TEV-SD1. The results show that all the plants inoculated with PVY^N remained symptomless whereas the plants inoculated with TEV-SD1 presented two types, namely resistant and

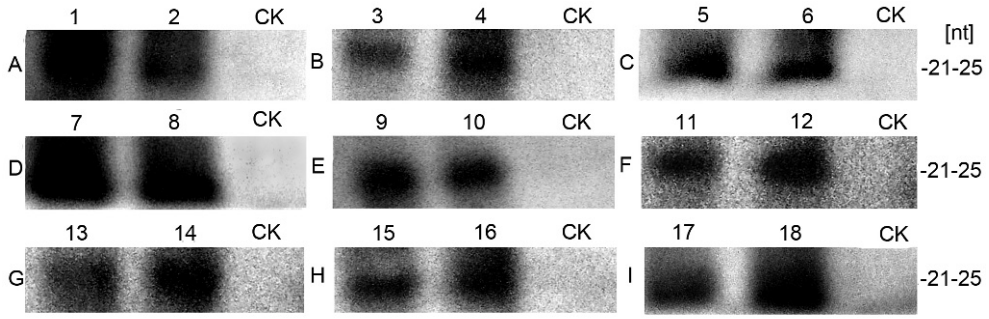

Fig. 2. Northern blot analysis of siRNAs from *Nicotiana benthamiana* plants with *Agrobacterium* mediated instantaneous expression. A to I - Accumulation of siRNAs from pR-NIB-S1, pR-NIB-S2, pR-HC-S3, pR-HC-S4, pR-CI-S5, pR-CI-S6, pR-HC-S7, and pR-NIB-S8 to pR-VPG-S9; 1 to 18 - *Nicotiana benthamiana* plants injected with recombinant binary vectors, respectively; CK - *Nicotiana benthamiana* plants injected with pROK II.

Table 2. Response of transgenic plants expressing the nine siRNA against PVY^N and TEV-SD1 infection (WT - wild tobacco NC89). Means ± SE, $n = 3$.

Transgene	Total number of plants tested	Number of plants resistant to PVY^N	PVY^N resistant [%]	Number of plants resistant to TEV-SD1	TEV resistant [%]
pR-NIB-S1	86	56	65.12 ± 1.57	36	41.86 ± 1.85
pR-NIB-S2	106	54	50.94 ± 2.14	54	50.94 ± 2.07
pR-HC-S3	104	12	11.54 ± 1.06	8	7.69 ± 0.56
pR-HC-S4	111	51	45.95 ± 0.92	45	40.54 ± 2.26
pR-CI-S5	80	14	17.50 ± 1.54	12	15.00 ± 1.25
pR-CI-S6	99	21	21.21 ± 1.68	18	18.18 ± 1.40
pR-HC-S7	114	48	42.11 ± 2.32	33	28.94 ± 1.65
pR-NIB-S8	86	46	53.49 ± 2.85	38	44.19 ± 2.18
pR-VPG-S9	93	21	22.58 ± 1.23	18	19.35 ± 1.38
WT	80	0	0	0	0

susceptible (Table 2). It was showed that the siRNA activity could indeed be affected by the mismatches between the siRNA and the target gene whereas the siRNA was proved to be effective. Furthermore, one resistant transgenic plant for each construct was randomly selected for further viral resistance analysis. The results show that PVY^N and TEV-SD1 were never detected in all T_1 plants produced from pR-NIB-S2-$T_0$35, pR-HC-S3-$T_0$2, pR-HC-S4-$T_0$1, pR-CI-S6-$T_0$12, and pR-NIB-S8-$T_0$27 whereas very few plants of the other groups showed symptoms of infection (Table 3). Our results indicate that shRNA-induced PVY^N and TEV-SD1 resistance could be inherited in T_1 generation plants.

Table .3 Resistance analysis of T1 transgenic plants challenged with PVY^N and TEV-SD1. Means ± SE, n = 3.

Transgenic plant	Number of plants tested	Resistance [%]
pR-NIB-S1-$T_0$24	70	97.22 ± 2.42
pR-NIB-S2-$T_0$35	66	100
pR-HC-S3-$T_0$2	54	100
pR-HC-S4-$T_0$1	70	100
pR-CI-S5-$T_0$6	63	97.03 ± 2.57
pR-CI-S6-$T_0$12	60	100
pR-HC-S7-$T_0$30	55	91.00 ± 2.75
pR-NIB-S8-$T_0$27	59	100
pR-VPG-S9-$T_0$10	60	98.33 ± 2.89
WT	20	0

Total RNA was extracted from partially resistant susceptible transgenic and wild plants to examine the expression of transgene-derived RNA transcripts. As expected, no hybridization signals were detected in the wild plants whereas special hybridization signals were observed in the transgenic plants from nine different transgenic lines. The transcript accumulation of the resistant transgenic plants was lower than that in the susceptible transgenic plants (Fig. 3). The result suggests that the resistance was inversely correlated with the RNA accumulation in transgenic plants.

The siRNAs were extracted from partially resistant transgenic plants. The analysis showed that siRNA (approximately 21 to 25 nt in length) hybridization

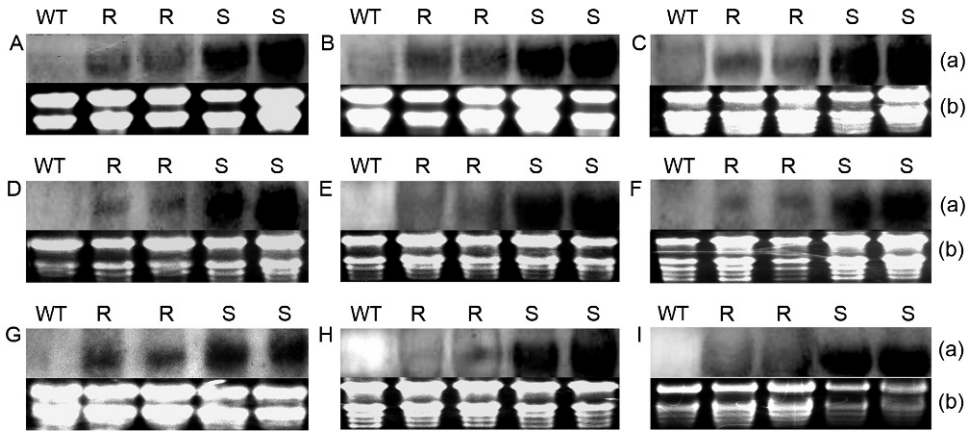

Fig. 3. Northern blot analysis of accumulation of transcripts in virus-inoculated transgenic plants. A to I - Accumulation of transcripts from pR-NIB-S1, pR-NIB-S2, pR-HC-S3, pR-HC-S4, pR-CI-S5, pR-CI-S6, pR-HC-S7, and pR-NIB-S8 to pR-VPG-S9; (a) - results of total RNA Northern blot, (b) - rRNA used to show that an equal amount of total RNA was loaded, WT - wild type plant, S - susceptible plant, R - resistant plant.

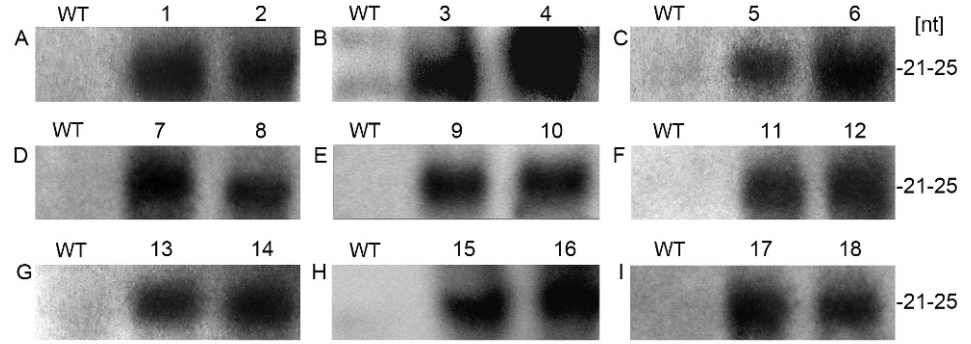

Fig. 4. Northern blot analysis of siRNAs in virus-inoculated transgenic plants. A to I - Accumulation of siRNAs from pR-NIB-S1, pR-NIB-S2, pR-HC-S3, pR-HC-S4, pR-CI-S5, pR-CI-S6, pR-HC-S7, and pR-NIB-S8 to pR-VPG-S9; 1 to 18 - resistant transgenic tobaccos with recombinant binary vectors, WT - wild tobacco NC89.

signals were detected in all resistant transgenic plants whereas no signal was observed in wild plants (Fig. 4). These results suggest that the viral resistance was indeed induced by RNA silencing.

The nine siRNAs and their target sites were analyzed to examine if the siRNA sequence characteristics affected silencing/resistance efficiency. Based on the comparison of the secondary structure stabilities of the antisense siRNA (Fig. 5), we found that S2, which has the highest minimum free energy value (ΔG^{0}_{37}= 5.3), did not induce the highest viral resistance whereas S8, which has the lowest minimum free energy values (ΔG^{0}_{37}= -3.7), did not induce the lowest viral resistance. No specific relationship was found between the minimum free energy of siRNAs and viral resistance (data not shown). This result suggests that the secondary structure stability of the antisense siRNAs slightly affected silencing efficiency. Based on the comparisons among the terminal nucleotide sequences of siRNAs, we found an undefined trend. When the 5' end base was U (S1 and S8), the efficiency of PVY^{N} resistance was higher. For instance, S1, whose 5' end bases were three consecutive Us, corresponded to the siRNA-mediated viral resistance at 65 %. Conversely, the proportion of resistant plants exceeded 40 % when the 5' end base was G (S2 and S7). When C was found at the 5' end (S3, S5, S6, and S9), the ratio of the resistant transgenic plants was less than 25 %. Thus, we speculate that 1 to 3 nt of the 5' end of the sense strand had a significant impact on siRNA activity, particularly U as the first base was favorable for silencing. Comparing the mismatched sites between the antisense siRNA and TEV-SD1 sequence (Table 1), we found that the mismatched sites significantly affected the viral resistance. The relative decline in the resistance rate between TEV-SD1 and PVY^{N} was 30 % when one to three mismatches were found in the 3' ends of S1, S3, and S7. The relative decline rate was approximately 10 to 30 % when one to two mismatches were located in the middle regions of S4, S5, S6, and S8. When the mismatches were observed in the 5' end of S2, the transgenic plants resistant to TEV-SD1 were also resistant to PVY^{N}. The results indicate that the mismatches may be more tolerated in the 5' end.

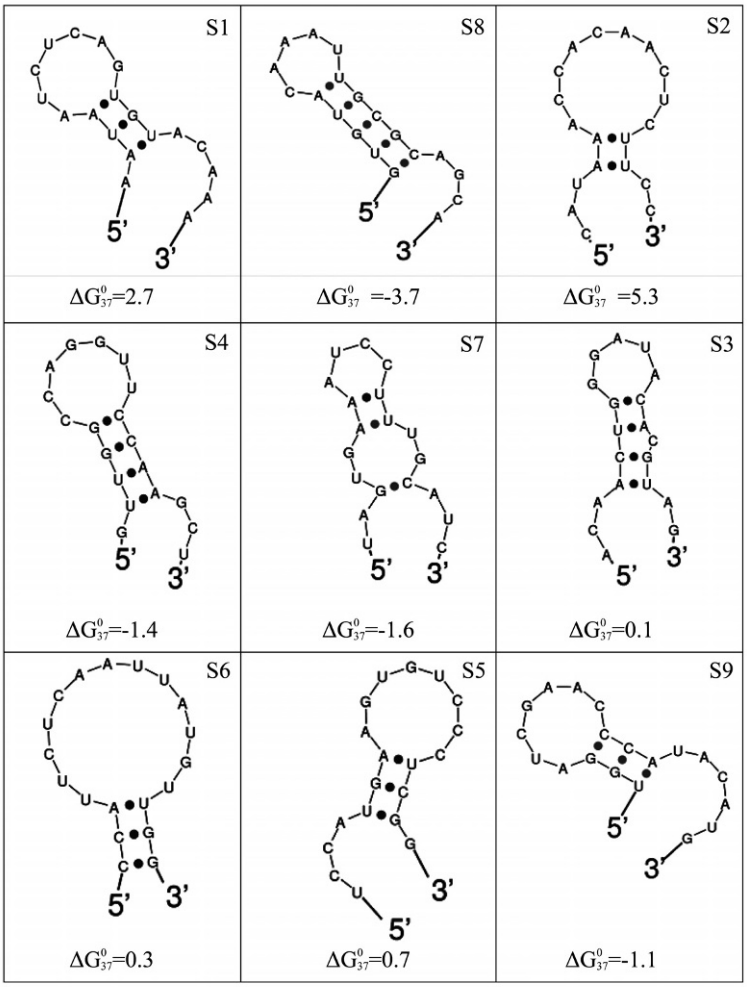

Fig. 5. The secondary structure stability of siRNA antisense chain. ΔG^{0}_{37} - minimum free energy.

Discussion

The dsDNAs have a variety of forming modes in different organisms (Zamore *et al*. 2002). For transgenic research, constructing hpRNA structures *in vitro* is the most effective way to produce dsRNAs. In mammals, when the length of dsRNA was more than 30 bp, antiviral/IFN pathways were excited to terminate protein synthesis of the entire system (Gil *et al*. 2000). However, endogenous expression of shRNAs, whose length was less than 30 bp, could trigger a related gene silencing (Yu *et al*. 2005). In plants, gene silencing is usually induced by the expression of long hpRNA. It was usually considered that long hpRNAs would induce higher silencing efficiency because they could produce more siRNAs. However, recent research showed that shRNAs could also effectively induce gene silencing (Qu *et al*. 2007). A single, small RNA accessible to RNA induced silencing complex (RISC), that could simultaneously target to multiple viruses, was sufficient to generate viral resistance (Duan *et al*. 2008). In this study, we selected nine 21 nt target sequences with high similarity between PVY^N and TEV-SD1 to construct shRNAs for viral resistance. The results showed that nine kinds of transgenic tobacco could exhibit resistance against both viruses and an effective single siRNA could induce more than 65.12 % silencing efficiency. We infer that the higher gene silencing efficiency might be mediated by secondary siRNAs produced by RNA-dependent RNA polymerases (RdRPs) in the RNA silencing as described by Sijen *et al*. 2007. Moreover, the utilization of shRNAs would minimize the risks of genome recombination and of field release during commercialization. It revealed that the shRNA strategy may have a more extensive application prospect.

Some studies indicated that not all qualified siRNA are equally effective (Holen *et al*. 2002). Many investigators have suggested that siRNA activity depended on many factors, such as the secondary structure of the sense siRNA (Patzel *et al*. 2005), the position of the target mRNA (Jiang *et al*. 2011), and the end base effect (Holen *et al*. 2002, Zeng *et al*. 2003). However, our research revealed that no relationship was observed between siRNA activity and the secondary structure of the siRNA (as shown in the χ^2-test of mRNA) (Chan *et al*. 2009). We speculate that RISC also exhibits a specific tolerance level to the secondary structure of siRNA in plants since the protein complexes involved in regulating the genes in mRNA and siRNA are similar (Ambros 2004). We also found that the position of the target mRNA within the full-length viral sequences significantly influenced siRNA activity which agrees with the results of previous studies (Holen *et al*. 2002, Molnar *et al*. 2005). We infer that some mRNA binding proteins might block the access of the special siRNAs to target mRNAs as shown by Qi *et al*. 2009. As for the end base effect, we speculate that it might be attributed to the rigorous criterion of the RISC-leading recognition between the siRNA and the target sequence.

RNA-mediated viral resistance is a sequence-dependent RNA degradation pathway. Some reports have indicated that the mismatch in 1 to 3 nt of the 5' end had no effect on siRNA activity whereas a significant effect was observed in the 3' end (Jackson *et al*. 2003, Pusch *et al*. 2003). However, in our research, silencing could also tolerate the mismatches in the middle region including the mismatch in the 10/11 position. We speculate that the viral resistance was also induced by the translational inhibition or sequestration of the target mRNA. However, the portion of these two ways in the silencing system was lower than that in mRNA degradation.

Reference

Ambros, V.: The functions of animal microRNAs. - Nature **431**: 350-355, 2004.

Bucher, E., Lohuis, D., Van Poppel, P.M., Geerts-Dimitriadou, C., Goldbach, R., Prins, M.: Multiple virus resistance at a high frequency using a single transgene construct. - J. gen. Virol. **87**: 3697-3701, 2006.

Chan, C.Y., Carmack, C.S., Long, D.D., Maliyekkel, A., Shao, Y., Roninson, I.B., Ding, Y.: A structural interpretation of the effect of GC-content on efficiency of RNA interference. - BMC Bioinformatics **10** (Suppl 1): S33, 2009.

Cogoni, C., Romano, N., Macino, G.: Suppression of gene expression by homologous transgenes. - Antonie Van Leeuwenhoek **65**: 205-209, 1994.

Donald, B.R., Martin, J.: Automated NMR assignment and protein structure determination using sparse dipolar coupling constraints. - Progr. nucl. magnetic Resonance Spectrosc. **55**: 101-127, 2009.

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

Fahim, M., Ayala-Navarrete, L., Millar, A.A., Larkin, P.J.: Hairpin RNA derived from viral *NIa* gene confer immunity wheat streak mosaic virus infection in transgenic wheat plants. - Plant Biotechnol. J. **8**: 821-834, 2010.

Fitchen, J.H., Beachy, R.N.: Genetically engineered protection against viruses in transgenic plants. - Annu. Rev. Microbiol. **47**: 739-763, 1993.

Gil, J., Esteban, M.: Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. - Apoptosis **5**: 107-114, 2000.

Guo, X.Q., Liu, S.E., Zhu, C,X., Song, Y.Z., Meng, X.B., Zheng, C.C., Wen, F.J.: RNA mediated viral resistance against potato virus Y (PVY) in transgenic tobacco plants. - Acta phytopathol. sin. **31**: 349-356, 2011.

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.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., Linsley, P.S.: Expression profiling reveals off-target gene regulation by RNAi. - Nat. Biotechnol. **21**: 635-637, 2003.

Jiang, F., Wu, B., Zhang, C., Song, Y., An, H., Zhu, C., Wen, F.: Special origin of stem sequence influence the resistance of hairpin expressing plants against PVY. - Biol. Plant. **55**: 528-535, 2011.

Lindbo J.A, Dougherty W. G.. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. - Virology **189**: 725-733, 1992.

Lindbo, J.A., Silva-Rosales, L., Proebsting, W.M., Dougherty, W.G.: Induction of a highly specific antiviral state in transgenic plant: Implications for regulation of gene expression and virus resistance. - Plant Cell **5**: 1749-1759, 1993.

Lucioli, A., Noris, E., Brunetti, A., Tavazza, R., Ruzza, V., Castillo, A.G., Bejarano, E.R., Accotto, G.P., Tavazza, M.: Tomato yellow leaf curl Sardinia virus Rep-derived resistance to homologous and heterologous Geminivirus occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. - J. Virol. **77**: 6785-6798, 2003.

Molnár, A., Csorba, T., Lakatos, L., Várallyay, E., Lacomme, C., Burgyán, J.: Plant virus-derived small interference RNAs originate predominantly from highly structured single-stranded viral RNAs. - J. Virol. **79**: 7812-7818, 2005.

Patzel, V., Rutz, S., Dietrich, I., Koberle, C., Scheffold, A., Kaufmann, S.H.: Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. - Natur. Biotechnol. **23**: 1440-1444, 2005.

Pusch, O., Boden, D., Silbermann, R., Lee, F., Tucker, L., Ramratnam, B.: Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA. - Nucl. Acids Res. **31**: 6444-6449, 2003.

Qi, X., Ba, F.S., Xie, Z.: Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA-dependent RNA polymerases in viral siRNA biogenesis. - PLoS One **4**: e4971, 2009.

Qu, J., Ye, J., Fang, R.: Artificial microRNA-mediated virus resistance in plants. - J. Virol. **81**: 6690-6699, 2007.

Ramesh, S.V., Mishra, A.K., Praveen, S.: Hairpin RNA-mediated strategies for silencing of tomato leaf curl virus AC1 and AC4 genes for effective resistance in plants. - Oligonucleotides **17**: 251-257, 2007.

Sijen, T., Steiner, F.A., Thijssen, K.L., Thijssen, K., Plasterk, R.: Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. - Science **12**: 244-247, 2007.

Szittya, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., Banfalvi Z., Burgyan J.: Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. - EMBO J. **22**: 633-640, 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 throughout gene silencing in plants. - Plant J. **27**: 581-590, 2001.

Xu, L., Song, Y.Z., Zhu, J.H.: Conserved sequences of replicase gene-mediated resistance to potyvirus through RNA silencing. - J. Plant Biol. **9**: 550-559, 2009.

Yan, P.Q., Li, L.J., Kang, H.: Cultivation transgenic tobacco against two viruses depending on RNAi. - China Bioeng. **27**: 27-31, 2007.

Yu, J.Y., Wang, T.W., Vojtek, A.B., Parent, J.M., Turner, D.L.: Use of short hairpin RNA expression vectors to study mammalian neural development. - Methods Enzymol. **392**: 186-199, 2005.

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

Zeng, Y., Cullen, B.R.: Sequence requirements for micro RNA processing and function in human cell. - RNA **9**: 112-123, 2003.

Zhu, C.X., Song, Y.Z., Yin, G.H.: Induction of RNA-mediated multiple virus resistance to potato Y, tomato mosaic and cucumber mosaic viruses. - J. Phytopathol. **157**: 101-107, 2009.