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ORIGINAL RESEARCH

Conserved Sequences of Replicase Gene-Mediated Resistance to *Potyvirus* through RNA Silencing

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Abstract Nuclear inclusion protein b (NIb) genes of three *Potato virus Y* isolates PVY-SD1 (O strain), PVY-SD4 (N strain), PVY-SD5 (NTN strain), and *Tobacco etch virus* isolate TEV-SD1 in Shandong Province were cloned and sequenced. Sequence analysis showed that the sequence homology of the entire NIb gene among the four viruses ranged from 65% to 95%. Hairpin RNA (hpRNA) constructs were designed based on five conserved regions derived from PVY-SD1 and introduced into tobacco plants. After asexual propagation, the transgenic plants were analyzed for resistance to PVY-SD1, PVY-SD4, PVY-SD5, and TEV-SD1. We obtained resistance ratios of 26.2%, 22.7%, 36.4%, 20.3%, and 21.7% to PVY-SD1. When inoculated with the PVY-SD5 virus, the transgenic plants had resistance ratios ranging from 2.4% to 15.9%, but no resistance at all to the other viruses, PVY-SD4 and TEV-SD1. No correlation was found between resistance of transgenic plants and the transgene copy numbers. Northern blot and small interfering RNA (siRNA) analysis demonstrated that the resistance was attributable to RNA silencing. Genetic analysis demonstrated that virus resistance was stably inherited in progeny T_1 and T_2. These results indicate that the siRNA molecules against conserved regions can confer virus resistance but are restricted to viruses with more than 90% sequence homology.

Keywords Conserved region · Hairpin RNA · NIb gene · *Potato virus Y* · RNA-mediated virus resistance

Potyviruses (genus *Potyvirus*, family *Potyviridae*) comprise the largest group of plant-infecting viruses that cause serious damage to crops such as potato and tobacco worldwide. Virus-resistant plants are in great demand to prevent the spread of and infection by viruses. Several approaches have been applied to obtain virus-resistant plants. Expression of virus genes or sequences in transgenic plants can confer protection from infection by a related virus. This form of resistance, introduced by Sanford and Johnson (1985), is known as pathogen-derived resistance. It has been shown that the resistance can occur at either the protein or RNA level, the latter being termed RNA-mediated virus resistance (Dougherty et al. 1994; Guo et al. 2001; Zhu et al. 2004). Virus-derived genes such as coat protein (CP) genes, replicase genes, and movement genes are effective in producing virus-resistant transgenic plants (Lindbo and Dougherty 1992; Carr et al. 1994; Lomonossoff 1995; Beachy 1997; Baulcombe 2002).

Mechanistically similar to posttranscriptional gene silencing, RNA-mediated virus resistance results in sequence-specific degradation of the viral RNA that carries the targeted sequence (Dougherty and Parks 1995; Baulcombe 1996; Zamore et al. 2000; Helliwell and Waterhouse 2003). The inverted-repeat construct of the

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target gene encodes a self-complementary hpRNA or double-stranded RNA (dsRNA). The dsRNA is processed into small interfering RNA (siRNA) 21 to 25 nucleotides (nts) in length by the RNaseIII enzyme Dicer and its homologs (Hamilton and Baulcombe 1999; Bernstein et al. 2001; Nykänen et al. 2001). These siRNAs are incorporated into the RNA-induced silencing complex (RISC) and guided to recognize complementary RNA for sequence-specific degradation (Hammond et al. 2000; Bernstein et al. 2001). Finally, the target mRNA is cleaved at a specific site corresponding to the siRNA antisense strand (Elbashir et al. 2001).

RNA-mediated virus resistance is affected by the sequence homology between the invading virus and the target transgenes (Mueller et al. 1995; Sonoda et al. 1999). Mismatches between the siRNA and its target could prevent the cleavage of the target RNA, reduce interference effects, and even make the siRNA inactive (Holen et al. 2002; Pusch et al. 2003). For transgenic pea lines expressing the nuclear inclusion protein b (NIb) gene of the *Pea seed-borne mosaic virus* (PSbMV) genome, Jones et al. (1998) concluded that 89% of the sequence homology between the NIb gene of the PSbMV and the isolates was borderline for resistance. In addition, several other studies have indicated that homology of a small target region rather than of the overall sequence may be critical for viral resistance (Sonoda et al. 1999; Balmori-Melian et al. 2002).

Potato virus Y (PVY) and *Tobacco etch virus* (TEV) are members of the *Potyvirus* genus. PVY has many variants that can be categorized into four groups according to their virulence and host response: PVY^{O}, PVY^{N}, PVY^{NTN}, and $PVY^{N}W$ (Glais et al. 2002). New variants will continuously emerge from the interaction of host and pathogen and the creation of new resistant varieties. The strategy of choice to confer resistance to PVY was the induction of RNA silencing by an ectopically expressed dsRNA that was conserved between *Potyviruses*. To generate RNA-mediated PVY-resistant transgenic plants and to test the possibility of generating multiple-virus-resistant plants using an RNA-mediated virus resistance approach, we designed hairpin RNA constructs based on five conserved regions of the NIb gene of *Potyviruses* and generated transgenic plants (Kamer and Argos 1984; Poch et al. 1989).

Materials and Methods

Materials

PVY-SD1, PVY-SD4, PVY-SD5 (O strain, N strain, and NTN strain) and TEV-SD1 were isolated and identified from infected leaves in the tobacco-growing area of Shandong Province. *Escherichia coli* strain DH5α and *Agrobacterium tumefaciens* strain LBA4404 were maintained in the laboratory. *Nicotiana tabacum* L. var. NC89 was utilized as the receptor in the transformation process.

Cloning of NIb Genes and Sequences Analysis

Viral RNAs from virus-infected leaves were extracted with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized from 2 μg of total RNA at 42°C for 1 h using M-MLV Reverse Transcriptase (Promega). DNA fragments of 1,557 bp containing the full coding region of the NIb gene were amplified by polymerase chain reaction (PCR) with 0.05 μg of the viral cDNAs as the template. PCR conditions were an initial denaturation step for 4 min at 94°C and 30 cycles of 1 min at 94°C denaturation step, 1 min at 56°C annealing step, and 2 min at 72°C extension step. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. Amplified DNA fragments were separated by 1% agarose gel electrophoresis and then recovered from the gel and purified. The purified DNA fragments were inserted into the pMD18-T vector (TaKaRa). After the DNA sequences of the cloned DNA fragments was sequenced, the homology of the sequence from the PVY-SD1 NIb gene was compared with PVY-SD4, PVY-SD5, and TEV-SD1 by DNAMAN. Five regions containing conserved sequences and sharing the highest identity among the isolates were located.

Construction of Plant Expression Vectors

Primers were designed based on the five located regions of the PVY-SD1 isolate. For the convenience of cloning, suitable restriction enzyme sites were added to the primers (Table 1). Fragments of 51 bp were amplified by PCR with the PVY-SD1 NIb as the template. The PCR-amplified fragments (F1, F3, F5, F7, and F9) were digested by *Xba*I and *Bam*HI, ligated to a compatibly digested binary vector pROKII, and then cloned into *E. coli* DH5α. The reconstructed plasmids were denoted PROKII-I, PROKII-II, PROKII-III, PROKII-IV, and PROKII-V. Then, fragments 75 bp in length were amplified. These fragments (F2, F4, F6, F8, and F10) were digested with *Sac*I and *Kpn*I and inserted into a similarly treated reconstructed plasmid PROKII-I, PROKII-II, PROKII-III, PROKII-IV, and PROKII-V, respectively. The resulting plasmids were denoted as PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V, respectively. Five inverted-repeat constructs were obtained which contained hpRNA fragment of NIb genes of PVY-SD1 (Fig. 1). Hairpin RNA molecules targeting these five conserved regions could be transcribed from these plasmids in transgenic plants.

Table 1 List of all forward and reverse primers

Sequence		Primers	Restriction enzyme
Region I	F1	P1: 5′-GCGC TCTAGACTCGGCATTTGGAACGGA-3′	*Xba*I
		P2: 5′-GCGC GGATCC ATT TGC AAGCTT CTCCTT-3′	*Bam*HI
	F2	P3: 5′-GCGC GAGCTCCTC GGC ATTTGGAACGGA-3′	*Sac*I
		P4: 5′-GCGC GGTACC AGCTTGCAAATTTCCTGTC-3′	*Kpn*I
Region II	F3	P5: 5′-GCGC TCTAGAGAT ACC TTGCTG GGTGG-3′	*Xba*I
		P6: 5′-GCGC GGATCCATTCTTTGA GTA AAATTGATTA-3′	*Bam*HI
	F4	P7: 5′-GCGC GAGCTCGATACCTTG CTGGGTGG-3′	*Sac*I
		P8: 5′-GCGC GGTACC AAATTG ATTATTGAAGTCATCA-3′	*Kpn*I
RegionIII	F5	P9: 5′-GCGC TCTAGATGGGTATACTGTGATGCTG-3′	*Xba*I
		P10: 5′-GCGC GGATCCATTGATTAAGTATGGAGTTAGT-3′	*Bam*HI
	F6	P11: 5′-GCGC GAGCTCTGGGTATACTGTGATGCTG-3′	*Sac*I
		P12: 5′-GCGC GGTACCTGGAGTTAGTGAACTATCAAA-3′	*Kpn*I
RegionIV	F7	P13: 5′-GCGC TCTAGAGGGAATAACAGTGGTCAGC-3′	*Xba*I
		P14: 5′-GCGC GGATCCCATGGCGAGGACGACCAT-3′	*Bam*HI
	F8	P15: 5′-GCGC GAGCTCGGGAATAACAGTGGTCAGC-3′	*Sac*I
		P16: 5′-GCGC GGTACCGACCATAAGAGAGTTGTCCA-3′	*Kpn*I
RegionV	F9	P17: 5′-GCGC TCTAGAGTGTTCTTTGTCAATGGTGA-3′	*Xba*I
		P18: 5′-GCGC GGATCCAAGAATACCCTCTTTATCCG-3′	*Bam*HI
	F10	P19: 5′-GCGC GAGCTCGTGTTCTTTGTCAATGGTGA-3′	*Sac*I
		P20: 5′-GCGC GGTACCCTCTTTATCCGGATTCACAG-3′	*Kpn*I

Plant Transformation

The recombinant binary vectors PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V were introduced into *A. tumefaciens* strain LBA4404 by the frozen-throwing method and eventually introduced into tobacco NC89 via leaf disk transformation. Transgenic plantlets were generated under the selection pressure of kanamycin and were further grown in a growth chamber at 25°C with a 16/8 h (light/dark) photoperiod (Sonoda et al. 1999).

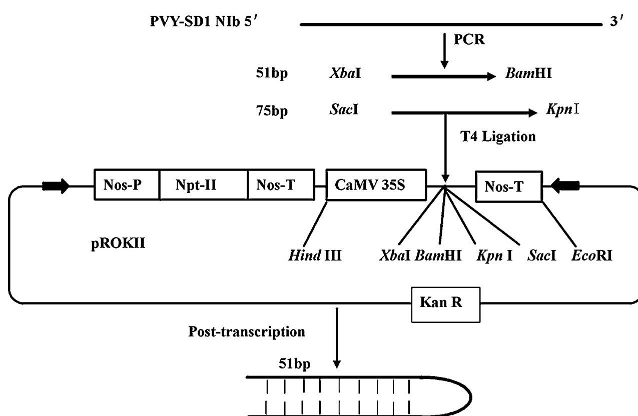

Fig. 1 Schematic representation of the construction of hpRNA expressing vector. DNA fragments of 51 and 75 bp of PVY-SD1 NIb were amplified and used to form inverted repeats. Upon transcription the inverted repeats is expected to form hairpin structures. Restriction sites: *Hind*III (total DNA digested with *Hind*III), *Xba*I, *Bam*HI, *Kpn*I, *Sac*I; *Npt-II* neomycin phosphotransferase II gene; *Nos-P* nopaline synthase promoter; *Nos-T* nopaline synthase terminator; *CaMV 35S* CaMV 35S promoter

Viral Resistance Assay of Transgenic Plants

To further assess virus resistance, transgenic plants were manually inoculated with the PVY isolates PVY-SD1 (O strain), PVY-SD4 (N strain), PVY-SD5 (NTN strain) and TEV-SD1 isolate. Inocula of these viruses were prepared by grinding virus-infected leaves diluted with phosphate buffer (PB, pH 7.4) at a ratio of 1:10 (*w*/*v*). Non-transgenic tobacco NC89 and transgenic plants transformed pROKII (vector control) were selected as the negative control. Inoculated plants were monitored daily for the appearance of symptoms after 2 to 3 weeks. Virus infection in plants was detected by indirect enzyme-linked immunosorbent assay (ELISA) 20 days after inoculation (Guo et al. 2001).

Southern Blot Analysis

To confirm the stable integration of transgenes into the genome and evaluate the transgene copy number, Southern blot analysis was performed. Total DNA of the

resistant plants and the susceptible plants was extracted using hexadecyltrimethylammonium bromide method (Zhu et al. 2004). Approximately 10 μg of digested DNA with the restriction enzyme *Hind*III were electrophoresed on a 1.0% (*w*/*v*) agarose gel and transferred to Hybond^{TM-}N+ membranes. Non-transgenic tobacco was used as the control. Southern blotting was conducted using the methods described by Sambrook et al. (1992). Transferred DNAs were then hybridized with [α-^{32}P] dCTP-labeled DNA fragments 75 bp in length amplified by PCR.

Northern Blot Analysis

Northern blot analysis was performed to ascertain transcript levels of the inserted transgenes. Total RNA was extracted with TRIzol reagent (Invitrogen) and dissolved in DEPC-H_2O. Ten micrograms of total RNA were electrophoresed on a 1.2% (*w*/*v*) agarose gel containing formaldehyde and transferred to Hybond^{TM-}N+ membranes with 20×SSC. The RNAs were hybridized with [α-^{32}P] dCTP-labeled probes identical to those used in the Southern blotting.

siRNA Analysis of Resistant Transgenic Plants

The presence of siRNA is a hallmark of the posttranscriptional gene silencing. To investigate the correlation between the presence of siRNAs and the resistance of transgenic plants, siRNAs were extracted from both resistant transgenic and non-transgenic plants. The siRNAs were extracted with 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% polyacrylamide gel containing 7 M urea, and then electrotransferred to a Hybond^{TM-}N+ membrane with the Trans-Blot (DYCP-40C) and fixed by UV cross-linking. The siRNAs were hybridized with a digoxigenin (DIG)-labeled (Roche Diagnostics) RNA probe at 42°C for 16 to 24 h. DIG-labeled RNA probes were synthesized by transcribing the target regions with T7 RNA polymerase according to the manufacturer's instructions. After hybridization, the membrane was treated with the anti-DIG alkaline phosphatase antibody and detected with CDP-Star according to the manufacturer's instructions (Roche Diagnostics).

Genetic Analysis of Resistant Transgenic Plants

The self-pollinated seeds were surface-sterilized and germinated on Murashige and Skoog media containing kanamycin. The kanamycin-resistant seedlings were green and showed vigorous growth. The transgenic plants were assayed for kanamycin resistance to check the segregation ratio. Then, the seedlings were transplanted to clay soil and grown in greenhouse for virus resistance analysis at the third or fourth leaf stage.

Results

Location of Conserved Sequences and the Sequence Homology Analysis

DNA fragments of 1,557 bp were amplified by PCR and inserted into the pMD18-T vector. The GenBank accession numbers were PVY-SD1 (EF470238), PVY-SD4 (EF470241), PVY-SD5 (FJ560596), and TEV-SD1 (EF470242). Comparative analysis of the sequence of the NIb genes among the four isolates showed the identities of the entire NIb gene ranged from 65% to 95%. Five regions containing the conserved sequence and sharing the highest nucleotide sequence identity were located. These regions shared 74.5% to 100% homology among these isolates,

Table 2 Sequences and identity of the conserved region used to design hairpin RNA vector

Target region	Nucleotide sequence(sense–strand) (5′–3′)	Nucleotide sequence identity (%)[a]			
		PVY-SD1	PVY-SD5	PVY-SD4	TEV-SD1
I	CTCGGCATTTGGAACGGATCATTGAAGGCAGAGCTCCGGTGTAAGGAGAAG	100	98	84.3	74.5
II	GATACCTTGCTGGGTGGTAAAGTGTGTGTTGATGACTTCAATAATCAATTT	100	96	84.3	76.5
III	TGGGTATACTGTGATGCTGATGGCTCACAGTTTGACAGTTCACTAACTCCA	100	96	84.3	84.3
IV	GGGAATAACAGTGGTCAGCCTTCTACTGTTGTGGACAACTCTCTTATGGTC	100	84.3	82.4	74.5
V	GTGTTCTTTGTCAATGGTGATGATCTGCTGATTGCTGTGAATCCGGATAAA	100	90.2	88.2	76.5
Overall		100	95	83.0	65.0

[a] Percentage against that of PVY-SD1 isolate

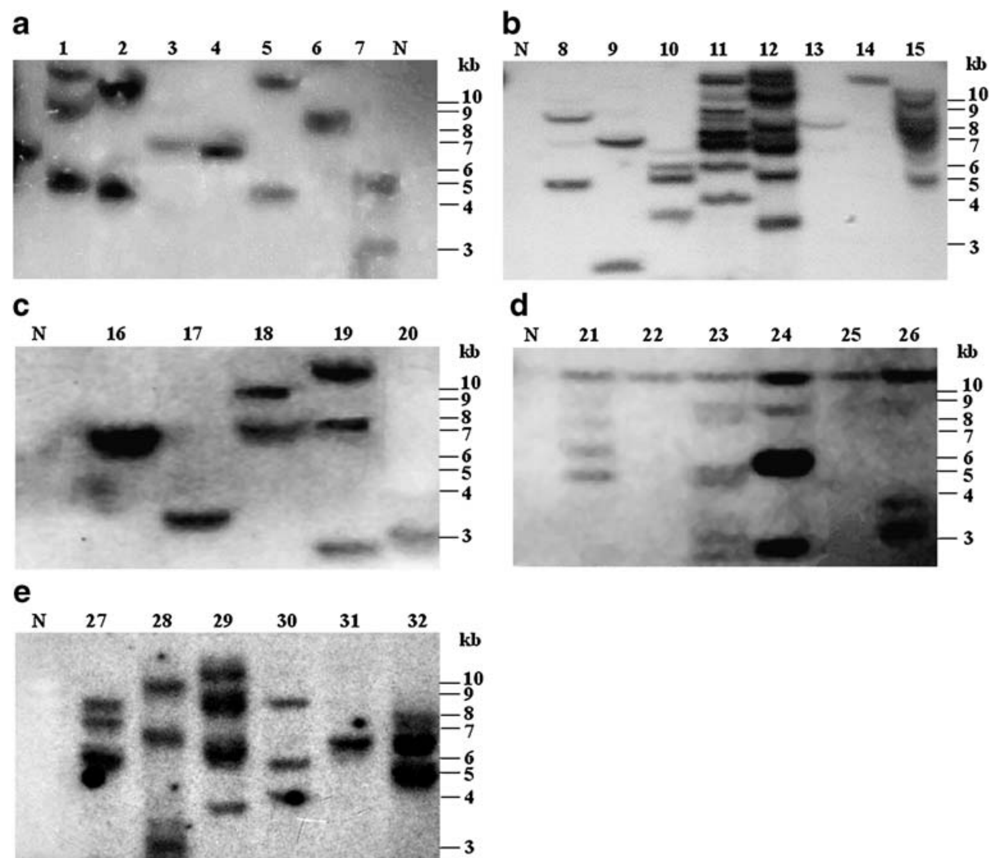

Fig. 2 Southern blot analysis of transgenic plants. **A**–**E** *Hind*III-digested genomic DNA from transformed lines PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V. Genomic DNA was separated on agarose gel, transferred to nylon membranes and probed with PVY NIb gene-specific probes. *Lanes N* non-transformed tobacco NC89, *Lanes 1 to 3*, *8 to 11*, *16 to 18*, *21 to 23*, and *27 to 29* show the results of susceptible phenotypes; *lanes 4 to 7*, *12 to 15*, *19 to 20*, *24 to 26*, and *30 to 32* show the results of resistant phenotypes

corresponding to nt 490 to 540, nt 583 to 633, nt 724 to 774, nt 919 to 969, and nt 1036 to 1086 of the PVY-SD1 NIb gene (Table 2).

Construction of Hairpin Plasmids and Plant Transformation

The target sequences 51 and 75 bp in length were amplified and purified on a 1.5% agarose gel. The 51- and 75-bp fragments were cloned into the XbaI/*Bam*HI and *Sac*I/*Kpn*I sites of pROKII, respectively, under the control of CaMV35S promoter. In this way, five recombinant plasmids of PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V were obtained. When the five recombinant plasmids were digested with *Xba*I and *Sac*I, about 125 bp fragments were obtained. The results showed that the lengths of fragments were identical to those expected (figure not shown). The binary vectors PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V were successfully constructed by cloning hpRNA into the plant expression vector of pROKII.

Table 3 Resistance of transgenic tobacco expressing PVY-SD1 NIb gene to *potyviruses*

Isolates	Transgenic plants[a]					
	PRIR-I	PRIR-II	PRIR-III	PRIR-IV	PRIR-V	N[b]
PVY-SD1	11/42	15/66	16/44	12/59	10/46	0/10
PVY-SD5	1/42	2/66	7/44	0/59	2/46	0/10
PVY-SD4	0/42	0/66	0/44	0/59	0/46	0/10
TEV-SD1	0/42	0/66	0/44	0/59	0/46	0/10

The values represent the number of resistant plants over the number of inoculated plants

[a] All transgenic plants that individually transformed plasmid constructs PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V

[b] Non-transformed tobacco plants

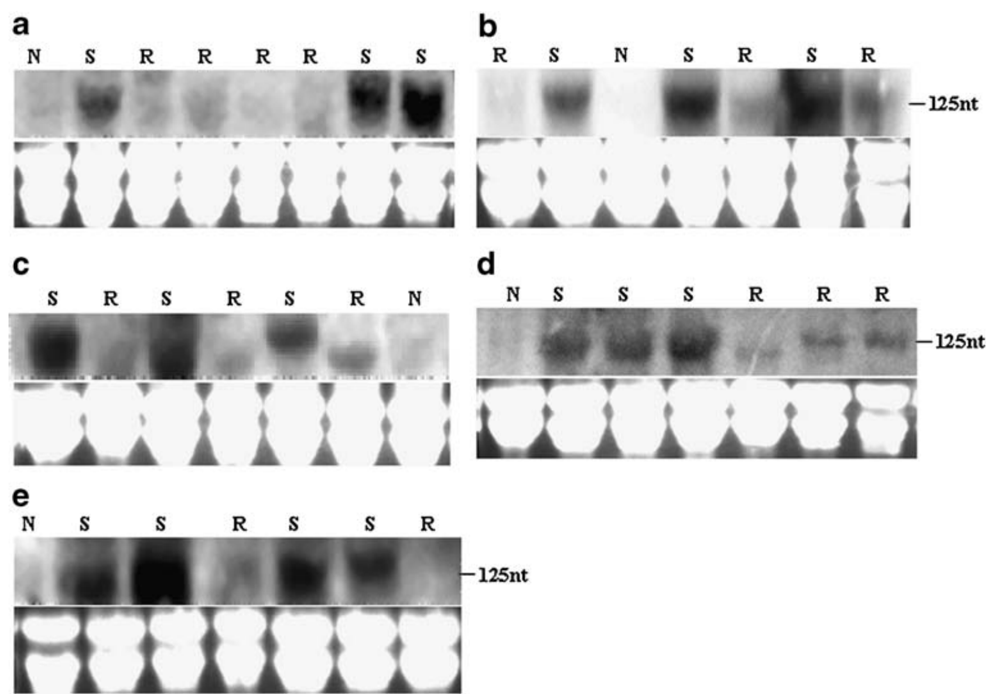

Fig. 3 Northern blot analysis of transgenic plants. **A**–**E** Total RNA (10 μg) extracted from different transgenic plants containing PRIR-I, PRIR-II, PRIR-III, PRIR-IV, and PRIR-V, respectively. The total RNA was subjected to denaturing gel electrophoresis, transferred to nylon membrane, and hybridized with the same probes used in the southern blot analysis. *Lanes R* represent total RNA from resistant transgenic tobacco plants. *Lanes S* represent total RNA from susceptible transgenic tobacco plants. *Lanes N* represent total RNA from non-transformed plants. The *lower panel* shows the loading level of each RNA sample by ethidium bromide staining

Transgenic lines contained PRIR-I, PRIR-II, PRIR-III, PRIR-IV, or PRIR-V, respectively. Kanamycin selection and PCR amplification were used to demonstrate the presence of the transgene in the transgenic plants, and all of transgenic plants appeared normal in morphology.

Southern Blot Analysis

Southern blot analysis showed no detection of any hybridization signals in non-transformed tobacco NC89. Lanes 1 to 3, 8 to 11, 16 to 18, 21 to 23, and 27 to 29 were loaded with digested DNA from the susceptible transgenic plants, while lanes 4 to 7, 12 to 15, 19 to 20, 24 to 26, and 30 to 32 were from the resistant transgenic plants. The number of DNA bands (insertions) varied for the different transgenic plants. Among lanes 3, 4, 6, 13, 14, 16, 17, 20, 22, 25, and 31, only one hybridization band was observed. This indicated that these transgenic plants contained a single copy, whereas the other transgenic plants had multiple copies (Fig. 2). These results verified that the foreign genes had been integrated into the plant genome, and no correlation was found between number of insertions and type of resistance.

Response of Transgenic Tobacco to PVY-SD1 Isolate

The transgenic tobacco plants transformed with PRIR-I, PRIR-II, PRIR-III, PRIR-IV, or PRIR-V were inoculated with PVY-SD1 at the third or fourth leaf stage. The PVY-SD1 isolate induced the typical symptom of viral infection on both non-transformed tobacco and vector control plants 20 days after the inoculation. Resistant plants were completely symptomless, and no virus content was detected by ELISA (data not shown). On the contrary, susceptible plants were severely affected with distinct vein-clearing and mosaic symptoms indistinguishable from those of the non-transformed tobacco. The ratio of resistant transgenic plants obtained for the five different transgenic lines were 26.2%, 22.7%, 36.4%, 20.3%, and 21.7%, respectively (Table 3).

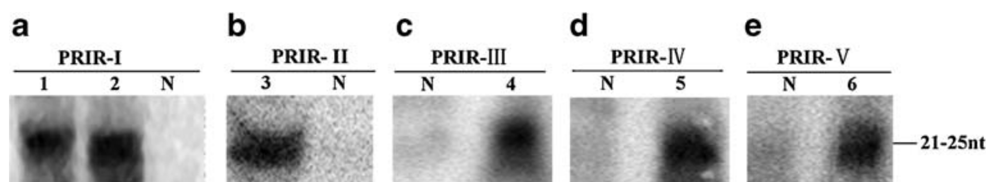

Fig. 4 Detection of NIb specific small interfering RNA (siRNA) from resistant transgenic tobacco plants. **A**–**E** Individual hybridizations with construct-specific probes. *Lanes 1 to 6* show the results of resistant phenotypes. Each lane corresponds to the same as follows: *lanes 1 and 2* PRIR-I; *lane 3* PRIR-II; *lane 4* PRIR-III; *lane 5* PRIR-IV *lane 6* PRIR-V. *Lanes N* non-transgenic tobacco plants

Table 4 Segregation of Kanamycin resistance in T_1 progeny

T_0 plants[a]	Kanamycin resistance in T_1 progeny			
	R/S[b]	Ratio	χ^2	*P* value
PRIR-I 4R	113/37	3:1	0.035	0.8507
PRIR-II14R	98/32	3:1	0.041	0.8401
PRIR-III 20R	101/37	3:1	0.153	0.6960
PRIR-IV 25R	110/40	3:1	0.141	0.7070
PRIR-V 31R	108/38	3:1	0.036	0.8504

[a] PRIR-I4R, PRIR-II14R, PRIR-III20R, PRIR-IV25R, and PRIR-V31R represent resistant transgenic plants of T_0 progeny

[b] R and S indicate kanamycin-resistant and susceptible T_1 progenies

Response of Transgenic Tobacco to other Two PVY Strains and TEV Isolate

Next, we investigated whether resistance to the other two PVY strains and TEV isolate could be achieved. The transgenic tobacco plants were inoculated against PVY-SD4 (N strain), PVY-SD5 (NYN strain), or TEV-SD1. For PVY-SD5, except for the transgenic tobacco containing PRIR-IV, the four other transgenic tobacco lines (PRIR-I, II, III, V) obtained resistance ratios of 2.4%, 3.0%, 15.9%, and 4.3%; all inoculated transgenic tobacco were susceptible to PVY-SD4 and TEV-SD1 (Table 3). In the case of the non-transformed tobacco, all of the isolates induced severe mosaic and/or necrosis of vein symptoms within 20 days of inoculation.

Northern Blot Analysis

To determine the accumulation and expression of transgene-derived RNA transcripts, Northern blot analysis was performed. As was expected, no hybridization signals were detected in the non-transformed plants. Susceptible transgenic plants accumulated a relatively high level of mRNA transcript. By contrast, the transgenic plants immune to virus infection showed a low level of mRNA transcript (Fig. 3). The transgene RNA accumulated less in resistant plants than it did in the susceptible plants, that is, there was an inverse correlation between the resistance and the amount of RNA accumulation in the transgenic plants.

siRNA Analysis in Resistant Transgenic Tobacco Plants

The occurrence of siRNA is one of the most important characteristics of posttranscriptional gene silencing. In the present study, the NIb-specific siRNAs were detected using a DIG-labeled RNA probes. The analysis showed that siRNA hybridization bands (approximately nt 21–25 in length) were detected in resistant transgenic plants (Fig. 4). No signal was observed in non-transgenic plants. These results suggested that RNA silencing was induced.

Resistant Inheritance of Transgenic Plants

When transgenic plants contain multiple copies, transgenes often induce genetic recombination in the progenies, exhibiting complicated heredity diversity. It is difficult to obtain homozygous transgenic plants. Stable inheritance can be obtained from low copy numbers of the foreign genes and endow transgenic plants with stable resistance. Therefore, we selected resistant transgenic lines PRIR-I4R, PRIR-II14R, PRIR-III20R, PRIR-IV25R, and PRIR-V31R that had one hybridization band (insertion) to study the segregation pattern and hereditary stability in T_1 and T_2 generation. Our results showed that the resistance segregation corresponded with Mendelian Segregation Law of single-site inserted inheritance. In the T_1 generation, the ratio of kanamycin-resistant plants to susceptible plants was nearly 3:1 (Table 4). In the T_2 generation, some transgenic plants had been stably homozygous, and the transgenic plants could not be differentiated at all; some transgenic plants still corresponded with the Segregation ratio of 3:1. In the virus-resistance analysis, all kanamycin-resistant plants in T_1 and T_2 generation were resistant to PVY-SD1 and PVY-SD5 but susceptible to PVY-SD4 and TEV-SD1 (the same as that of its parent T_0 generation; data not shown). Northern blotting showed that there was accumulation of siRNA in T_2 generation (Fig. 5). The results above showed that RNA-mediated virus resistance can be stably inherited.

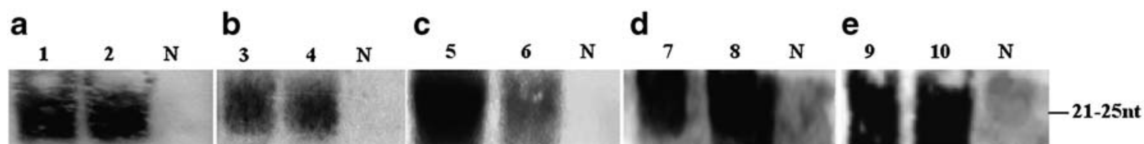

Fig. 5 Detection of siRNA in T_2 progeny of transgenic plants. **A**–**E** Results of resistant phenotypes. *Lanes N* non-transgenic tobacco plants. *Lanes 1 and 2*, *3 and 4*, *5 and 6*, *7 and 8*, and *9 and 10* represent the results of T_2 progenies from resistant (PRIR-I4R, PRIR-II14R, PRIR-III20R, PRIR-IV25R, and PRIR-V31R) T_0 lines, respectively

Discussion

In previous studies, virus-resistant transgenic plants have been generated by the expression of viral coat protein genes in the plants (Abel et al. 1986; Golemboski et al. 1990). Resistance to multiple viruses may be achieved by expressing different plant viral coat protein genes in the plants (Kaniewsli et al. 1990; Prins et al. 1995; Fuchs et al. 1997). The expression of coat protein genes from different viruses usually results in the exhibition of different types of resistance (Golemboski et al. 1990; Kouassi et al. 2006; Krubphachaya et al. 2007). However, the coat protein genes may also recombine with other heterogeneous viruses in nature and produce dangerous viruses that are transmittable and prevalent (Jan et al. 2000; Zhou and Li 2000; Bucher et al. 2006). As there is no transgenic mRNA accumulation in transgenic plants, RNA-mediated virus resistance has the advantage of biosafety and can produce multiple virus-resistant transgenic plants (Lindbo and Dougherty 1992). In our experiment, we designed hpRNA constructs based on the five conserved regions of the NIb gene of the PVY-SD1 virus and generated transgenic plants. The transgenic plants were resistant to infection by the PVY-SD1 virus at different ratios, with the highest ratio being 36.4% of complete resistance.

RNA-mediated virus resistance is a sequence-dependent RNA degradation pathway, but there is no perfectly positive correlation between the percentage of resistant transgenic plants, the identity of the transgene, and the target sequence. The sequence of the target mRNA is a very important determinant of siRNA efficacy and might play an essential role in maintaining or discarding the guide strand in RISCs (Amarzguioui and Prydz 2004; Hohjoh 2004; Liao et al. 2008). Our previous study demonstrated a significant difference between the 5′ and 3′ ends of the coat protein gene of *Potato virus Y*-mediated virus resistance (Li et al. 2007). In siRNA-mediated gene silencing, it was observed that siRNA sequences have a nonrandom distribution along the length of the viral genome, suggesting there are hot spots for virus-derived siRNA generation (Molnár et al. 2005) and not all the positions of siRNA molecule contribute equally to target recognition. Holen et al. (2002) synthesized 14 siRNAs against different sites on the same target mRNA (human Tissue Factor) to demonstrate striking differences in silencing efficiency. Only a few of the siRNAs resulted in a significant reduction in target gene expression. McManus and Sharp (2002) designed siRNAs targeting the different regions of target mRNA, including the coding region, 3′ untranslated region (3′ UTR), and 5′ untranslated region (5′ UTR), and determined that the suppression effects varied significantly, with no effects in coding regions. The reason for these results may be a “position effect”, meaning the secondary structure of the mRNA or binding of specific protein on the mRNA blocks the access to the siRNA molecules (Holen et al. 2002; Westerhout and Berkhout 2007; Gredell et al. 2008).

RNA-mediated virus resistance is homology-dependent gene silencing. It is possible to obtain transgenic plants resistant to multiple viruses using template DNA fragments with high identity selected from different viruses (strains). Our results have shown that transgenic plants exhibited resistance to two strains, PVY-SD1 and PVY-SD5, which led to speculation about producing transgenic tobacco that is resistant to multiple viruses (strains). Considering the high genetic variability of the viruses, siRNA-targeting of conserved areas of the viral genome can protect the plants from infection by different viral strains and escape mutants (Liu et al. 2005; Lyn O’Brien 2007).

RNA-mediated virus resistance seems to be effective only against viruses with closely related sequences (Baulcombe 1996; Jones et al. 1998; Bau et al. 2003). The threshold similarities between the transgene and the infecting virus appear to vary case by case, and the lowest reported sequence identity is 86.6% (Nomura et al. 2004). Moreno et al. (1998) showed that when designing transgenes for *Potyvirus* resistance, it is important to select for regions of nearly 90% identity between strains to obtain a wider resistance. Our results showed that the breadth of the RNA-mediated virus resistance was generally restricted to viral strains with about 90% sequence homology to the introduced transgenes. These results will contribute to the elucidation of the mechanism for RNA-mediated virus resistance and offer an effective strategy for cultivating multi-virus-resistant plants.

Moreover, the small segments of the transgene can minimize the risks of recombination, transcapsidation, synergism, or complementation and also avoid the potential risks of field release and commercialization.

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