DOI: 10.1007/s10535-014-0422-9 BIOLOGIA PLANTARUM 58 (3): 575-581, 2014

Different target genes and chimeric-gene hairpin structures affect virus resistance mediated by RNA silencing in transgenic tobacco

X. XIE, Y. SONG, X. LIU, S. WANG, 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

Two types of hairpin RNA (hpRNA) constructions were designed using a chimeric gene formed from two genes, the coat protein (CP) gene or the silencing suppressor gene, from the *Cucumber mosaic virus* (CMV) and the *Potato virus Y* (PVY^N), respectively; one type generated a single hairpin structure, whereas the other formed a two-hairpin structure. Four constructs, pDCPSH (double CP gene fragments, single hairpin), pDCPDH (double CP gene fragments, double hairpins), pHC2bSH (two silencing suppressor gene fragments, single hairpin), and pHC2bDH (two silencing suppressor gene fragments, double hairpins), were individually introduced into tobacco plants. A transcript analysis demonstrates that the small interference RNA (siRNA) processing efficiency was greater with the double-hairpin construct than with the single-hairpin construct, although the expression of their target genes were similar. A viral resistance assay shows that the transgenic tobacco plants effectively resisted a mixed infection of CMV and *Potato virus Y* (PVY^N) and that pDCPDH exhibited the highest silencing efficiency. The accumulation of siRNA in the inoculated transgenic plants expressing different hairpin structures was similar. A genetic analysis reveals that viral resistance in the transgenic plants was stably inherited from the T_0 to T_1 generation. A transcript analysis and a viral resistance assay indicate that the double-hairpin structure of the same target sequences tended to produce more siRNA before the virus inoculation and thus strengthened RNA-mediated viral resistance.

Additional key words: coat protein, *Cucumber mosaic virus*, dual-virus resistance, *Nicotiana tabacum*, *Potato virus Y*, silencing suppressor.

Introduction

RNA silencing is a sequence-specific process of mRNA degradation (Hannon 2002). In this process, small interference RNA (siRNA) derived from a virus guides the homologous RNA degradation of invading viruses, thus endowing transgenic plants with viral resistance which is referred to as RNA-mediated virus resistance (RMVR) (Prins *et al*. 2008). Previous studies have shown that siRNA has a major role in RNA silencing. The hairpin RNA (hpRNA) precursor observed in plants is the most successful expression cassette for the double-stranded RNA (dsRNA) expression to date, particularly siRNAs with 21 to 25 nucleotides (Watanabe 2011).

Under field conditions, multiple viruses may attack plants simultaneously, and the synergism of the viruses may exacerbate disease hazards. Therefore, it is particularly urgent and important to create transgenic crops with multi-viral resistance. To expand the viral resistance range, Prins *et al.* (2008) recommended using a chimeric gene that was spliced with nucleic acid fragments of different viruses or different strains of the virus to build a multi-virus (multi-strain) chimeric gene expression vector for plant transformation. Through this strategy, transgenic plants with multivirus resistance have been obtained in tobacco, *Nicotiana benthamiana*, melon, potato, and tomato (Boucher *et al*. 2006, Zhu *et al*. 2009, Wu *et al*. 2010, Lin *et al*. 2011, Arif *et al*. 2012). However, previous

Received 4 June 2013, *last revision* 25 September 2013, *accepted* 25 October 2013.

Abbreviations: CMV - *Cucumber mosaic virus*; CP - coat protein; hpRNA - hairpin RNA; PVY - *Potato virus Y*; siRNA - small interference RNA.

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

* Corresponding authors; fax: (+86) 0538 8248696, e-mail: zhchx@sdau.edu.cn ; fjwen@sdau.edu..cn

studies have indicated that the construction of chimeric-genes is repetitious, and the method multi-resistant efficiency is low (Kung *et al*. 2009, Zhu *et al*. 2009, Lin *et al*. 2011).

Potato virus Y (PVY) and *Cucumber mosaic virus* (CMV) are two serious viruses that affect tobacco production. PVY CP and CMV CP wrap the viral nucleic acid and play a role in nucleic acid protection (Audy *et al*. 1994, Takeshita *et al*. 2001). PVY HC-Pro is a viral aphid accessory protein (Urcuqui-Inchima *et al*. 2001), and CMV 2b is a viral operation protein (Praveen *et al*. 2008), both of which are silencing suppressors. In the current study, single-hairpin or double-hairpin vectors were constructed using CMV and PVY^N CP genes or suppressor genes. Plants that were resistant against the two viruses were obtained by transforming tobacco plants using these vectors. Viral resistance was correlated with different types of hairpin structures and target genes. The results provide useful information for the future development of transgenic plants with highly efficient resistance to multi-viral infection.

Materials and methods

The CP and the HC-Pro genes of PVY-SD (EU182576), and the CP and the 2b genes of CMV-SD (AY792596, EU414798) were selected as the target sequences. The primers of the hpRNA target sequences in this study were designed using the *Primer5* software (Table 1 Suppl.).

Previous studies have shown that a length of 300 bp can effectively initiate gene silencing (Jan *et al.* 2000, Kotlizky *et al.* 2001). The cDNA fragments of PVY CP (300 bp; F1), CMV CP (300 bp; F2), PVY HC-Pro (300 bp; F5), and CMV 2b (300 bp; F6) were obtained by PCR amplification using the corresponding primer pairs, and then inserted into the cloning vector pUC19 to obtain the chimeric-gene fragments of 600 bp of the CP genes (F3) or the suppressor genes (F7). The F4 and F8 fragments were amplified by the corresponding primer pairs using the F3 or F7 fragments as templates. The peroxidase (POD) gene intron fragment (F9) was amplified with primers P17 and P18. Fragments F3+F9+F4 and F7+F9+F8 were inserted into the plant expression vector, pROKII, to obtain the single-hairpin plant expression vectors pDCPSH and pHC2bSH, respectively (Fig. 1*A*).

The cDNA fragments PVY CP (300 bp; F10 and F11) and CMV CP (300 bp; F12 and F13) and the POD gene intron fragment (F14) were obtained from their corresponding primer pairs. These fragments were inserted into the cloning vector, pBSK, to obtain the recombinant fragments hpPVYCP (F10+F14+F11) and hpCMVCP (F12+F14+F13), produced by the cloning vector, pEASY-T, as were the recombinant fragments, hpPVYHC (F15+F19+F16) and hpCMV2b (F17+F19+F18). These recombinant fragments were digested by restriction enzymes *Bam*HI/*Xba*I and *Sac*I/*Kpn*I, respectively, and inserted into the plant expression vector, pROKII, to obtain the double-hairpin expression vectors pDCPDH and pHC2bDH, respectively (Fig. 1*B*).

The recombinant plant expression vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404 using the frost thawing method and introduced into tobacco (*Nicotiana tabacum* L.) cv. NC89 through the leaf disk transformation method (Horsch *et al*. 1985). The transgenic plants were verified using PCR detection.

The transgenic plants were infected with a mixture of CMV and PVY^N, and wild type tobacco plants were used as negative control. Virus infection in plants was detected by an indirect enzyme-linked immunosorbent assay (ELISA) three weeks after the inoculation (Guo *et al*. 2001). Three replicates were used in this study.

Total RNA was extracted with a *Trizol* reagent (*Invitrogen*, Carlsbad, USA). siRNA was extracted using a *PureLink*™ miRNA isolation kit (*Invitrogen*).

mRNA expression in the transgenic plants was examined by real-time quantitative PCR (RT-qPCR) (Chen *et al*. 2005). Total RNA was converted to cDNA using a *Prime-Script* RT-qPCR reagent kit (*TaKaRa*, Otsu, Shiga, Japan) primed with a RT-qPCR primer (Table 2 Suppl.). The RT-qPCR was performed using a *SYBR PrimeScript* RT-qPCR kit (*TaKaRa*) on a *CFX96* real-time system (*Bio-Rad*, Shanghai, China). All samples were run in triplicate.

For Northern blot and siRNA analyses, probe preparation and blot hybridisation were performed using a *DIG Northern Starter* kit (*Roche*, Mannheim, Germany).

Total DNA was extracted from resistant plants by the cetyltrimethylammonium bromide (CTAB) method using 2 % (m/v) CTAB (Huang *et al.* 2000). DNA (100 μg) was digested with *Eco*RI. Southern blot was performed using a *DIG High Prime* DNA labelling and detection starter kit I (*Roche*), following the manufacturer's instructions. T_1 seeds of the transgenic plants with one-loci detected by Southern bloy were germinated on a Murashige and Skoog (MS) medium containing 100 mg dm^{-3} kanamycin for segregation analysis to validate the Southern blot results. Wild type NC89 tobacco seeds germinated on the medium without kanamycin were used as control. These T_1 plants were used in the subsequent virus resistance tests.

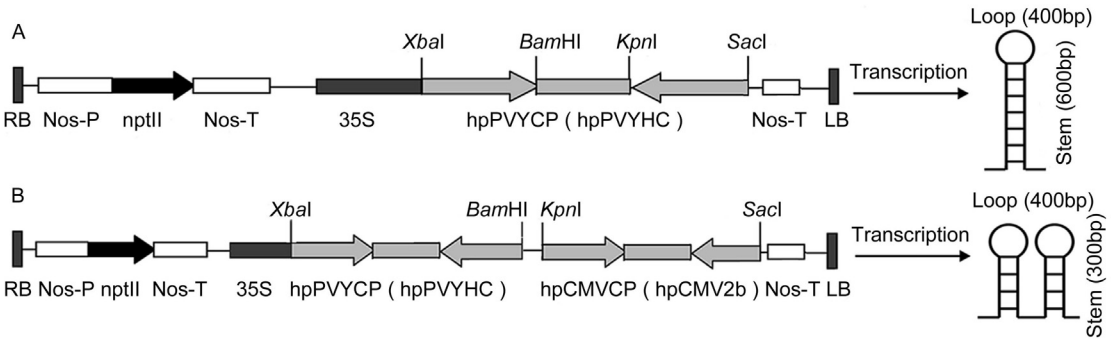

Fig. 1. The construction strategy of plant expression vectors: *A* - the construction of expression vectors pDCPSH and pHC2bSH containing a single-hairpin; *B* - the construction of expression vectors pDCPDH and pHC2bDH containing a double-hairpin. RB - T-DNA right border, LB - T-DNA left border, Nos-P - nitrous oxide systems (Nos) promoter, npt II - neomycin phosphotransferase gene, Nos-T - Nos terminator, 35S - *Cauliflower mosaic virus* (CaMV) 35S promoter, hpDCP - the inverted repeat sequence (IRS) of chimeric coat protein (CP) genes of PVY^N and CMV, hpHC2b - the inverted repeat sequence (IRS) of chimeric suppressor genes of PVY^N and CMV, hpPVYCP - IRS of the CP gene of PVY^N, hpPVYHC - IRS of the HC-Pro gene of PVY^N, hpCMVCP - IRS of the CP gene of CMV, hpCMV2b - IRS of the 2b gene of CMV.

Results

The target sequences were amplified by PCR using the corresponding primer pairs. The amplified fragments were cloned into the pUC19, pBSK, and pEASY-T cloning vectors. The reverse chimeric gene structures were constructed using these cloning vectors and were then inserted into the plant expression vector, pROKII. The plant expression vectors harbouring a single-hairpin of the dual-virus chimeric-gene or a double-hairpin of the dual-virus genes pDCPSH, pHC2bSH, pDCPDH, and pHC2bDH were obtained (Fig. 1), and the vectors were introduced into tobacco NC89. Through kanamycin selection and PCR detection, 106, 111, 104, and 108 plants from pDCPSH, pDCPDH, pHC2bSH, and pHC2bDH were obtained, respectively. The transformed plants grew normally, and there was no significant difference between the wild type (WT) and transgenic plants.

We measured the expression of the target genes using RT-qPCR. The results showed that the *p*-value was lower than 0.01 between pDCPDH and pDCPSH, and that the *p*-value was also lower than 0.01 between pHC2bDH and pHC2bSH. There was no significant difference in the target gene expression between the transgenic plants which were transformed with the single-hairpin structure or double-hairpin structure vectors using the same target genes. Northern blot was performed to examine the accumulation of siRNA in the transgenic plants before the inoculation. Because the total amount of siRNA from each individual plant was small, 20 transgenic plants expressing the same vector were used to extract the siRNA, and the concentrated sample was used for the Northern blot analysis. It shows that the accumulation of siRNA in the transgenic plants transformed with the double-hairpin structure vector was significantly greater than in those transformed with the single-hairpin structure vector using the same target genes (Fig. 2).

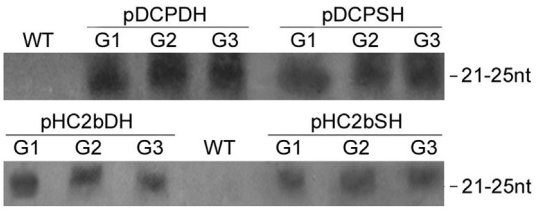

Fig. 2. The Northern blot analysis of siRNA processing efficiency from transgenic plants. Northern hybridised probe 1 were chimerical CP genes of PVY^N and CMV for marking (the length of 600 bp); Northern hybridised probe 2 were chimerical suppressor genes of PVY^N and CMV for marking (the length of 600 bp). WT: Wild type plants (NC89) without viral infection; G1, G2, G3 - group 1, group 2, and group 3; 10 μg of siRNAs was extracted from 20 transgenic plants per group.

We manually inoculated the transgenic tobacco plants of the T_0 generation, which were validated by PCR, and the non-transgenic plants (wild type NC89) with a CMV and PVY^N mixture. After three weeks, all of the wild tobacco plants showed the typical symptoms of CMV and PVY^N infections. In comparison, both resistant and susceptible plants were observed among the transgenic plants. No viral infection symptoms were observed in the resistant plants, and the ELISA assay did not detect the accumulation of the virus in these plants (data not shown). In contrast, the susceptible plants displayed symptoms of distinct vein-clearing, mosaic leaves, necrotic vein, and wilted sprout that were similar to those displayed by the infected wild type plants (data not shown). The percentage of resistant plants in the pDCPSH, pDCPDH, pHC2bSH, and pHC2bDH transgenic plants were 47.08, 63.25, 25.78, and 37.98 %, respectively (Table 1). These results show that the

expression vectors (pDCPSH and pDCPDH) that targeted the CP gene provided higher resistance efficiency in the transgenic plants (Table 1). The resistance might be affected by different functional genes. The expression vectors (pDCPDH and pHC2BDH) with the double-hairpin structure provided greater resistance efficiency than did those with the single-hairpin structure (Table 1) suggesting that the resistance ratio was affected by differences in the structures.

Table 1. The response of transgenic plants to CMV and PVY^N infection. Data represent the average of three replicates ± SE.

Plants	Number of infected plants	Number of resistant plants	Resistant plants [%]	Average ratio [%]
pDCPSH	40	19	47.50	47.08±1.91
	35	17	48.57	
	31	14	45.16	
pDCPDH	40	24	62.50	63.25±0.75
	38	25	63.16	
	33	21	63.64	
pHC2bSH	40	11	27.50	25.78±1.75
	35	9	25.71	
	29	7	24.14	
pHC2bDH	39	15	38.46	37.98±1.87
	36	13	36.11	
	33	13	39.39	
WT	50	0	0	0.00

The total RNA analysis of the inoculated resistant plants shows that no exogenous hybridisation signals were detected in the WT plants, whereas specific hybridisation signals were observed in the transgenic plants transformed with four different constructs (Fig. 3). The accumulation of viral RNA in the resistant plants was lower than that in the susceptible transgenic plants (Fig. 3*A*). The siRNA analysis shows that siRNA (approximately 21 to 25 nt in length) hybridisation signals were detected in all of the inoculated transgenic plants, whereas a signal was not observed in the inoculated wild type plants (Fig. 3*C*). These results suggest that the viral resistance was indeed induced by RNA silencing.

Total DNA was extracted from some resistant plants transformed with pDCPDH, pHC2bSH, pHC2bDH, and pDCPSH and from WT tobacco NC89 as the control. Southern blot shows that hybridisation signals were detected in all of the resistant transgenic plants, but no signal was observed in the WT plants (Fig. 4). This result demonstrates that the exogenous genes were integrated into the tobacco genome. The resistant T_0 generation plants which showed one band in the Southern hybridisation analysis (from lines of pDCPSH-28R, pDCPDH-48R, pHC2bSH-72R, and pHC2bDH-29R), were selected for the kanamycin segregation analysis. The segregation result shows that the ratios of resistant to non-resistant plants were 3:1 in the pDCPDH-48R, pHC2bSH-72R, and pHC2bDH-29R lines, whereas in pDCPSH-28R, it showed the ratio of 15:1.

Approximately 15 transgenic tobacco plants containing each vector were grown in a greenhouse and were inoculated with the CMV and PVY^N mixture. Three replicates were used in this study. After three weeks, there were no symptoms of viral diseases on most of the plants, and the percentage of resistance plants reached

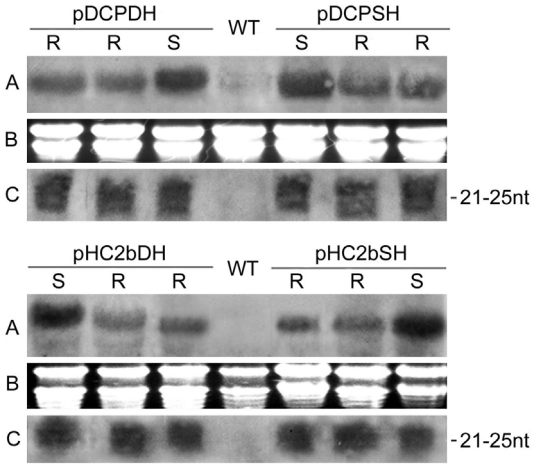

Fig. 3. The Northern blot analysis of inoculated transgenic plants. Probe 1 and probe 2 were used for hybridisations. *A* - the results of total RNA Northern blot; *B* - rRNA was used to show that the equal amount of total RNA was loaded; *C* - the results of siRNA assay. R - resistant, S - susceptible, WT - wild type (NC89), plants.

Table 2. The genetic analysis of resistant transgenic tobacco plants with one insert band. Data represent the average of three replicates ± SE. +CK - plants rooted in a medium containing 100 mg dm^{-3} kanamycin, -CK - plants rooted in a medium without kanamycin, R - resistant, S - susceptible, I - infected.

Plants	Kanamycin resistance R/S	ratio	CMV and PVY resistance R/I	ratio [%]
T_1- pDCPDH -48R	177:66	3:1	53/54 55/56 51/51	98.79±0.62
T_1- pHC2bSH -72R	188:71	3:1	54/55 50/51 53/54	98.12±0.09
T_1- pHC2bDH-29R	173:64	3:1	52/53 55/56 51/52	98.13±0.08
T_1- pDCPSH-28R	161:13	15:1	55/57 49/51 47/48	96.83±0.81
WT + CK	0:77	-	-	-
WT - CK	49:6	-	-	-

95 % or greater, whereas viral disease symptoms appeared in all of the inoculated WT plants (Table 2). This result indicates that the resistance was stably inherited by the T_1 generation.

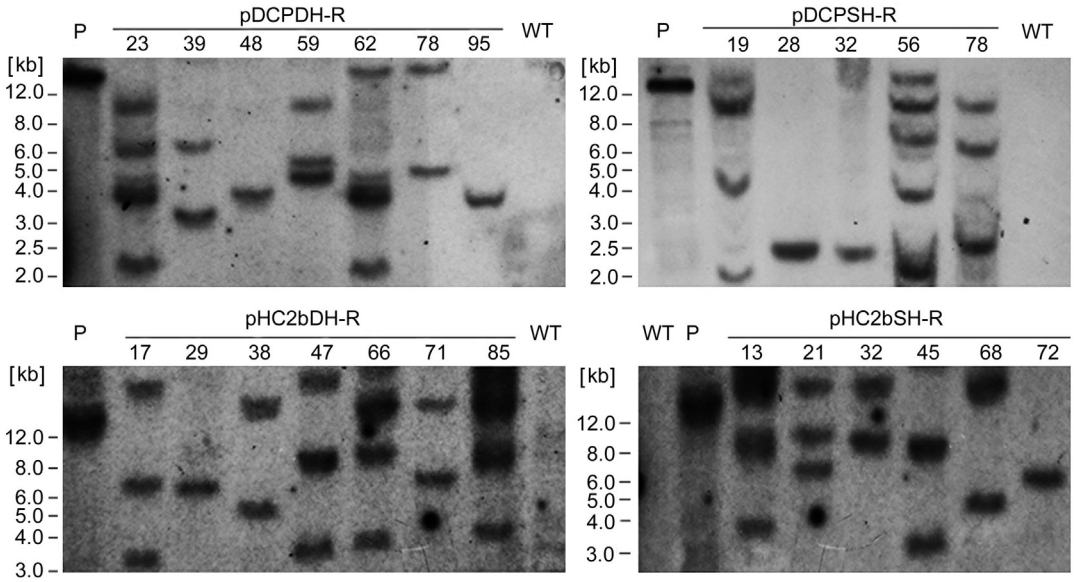

Fig. 4. The Southern blot analysis of T_0 resistant plants. Total DNA from resistant plants transformed with pDCPSH, pDCPDH, pHC2bSH, and pHC2bDH were hybridised with Southern blot probes. Southern blot probe 1 and probe 2 were PVY CP300 (the length of 300 bp) or PVY HC-Pro300 (the length of 300 bp), respectively. WT - wild type plants (NC89), P - plasmid containing expression vector.

Discussion

To obtain viral-resistant transgenic plants using the RMVR strategy, the construction of an efficient hpRNA expression vector is vital (Stoutjesdijk *et al*. 2002). The silencing efficiency of hpRNA generated by the variation in the stem length (Hammond *et al*., 2000), the loop position (Yoshinari *et al*. 2004), the target gene (Yoshinari *et al*. 2004), and the suitable promoter (Zhang *et al*. 2012) has been reported. However, most previous studies were focused on a single construction of a viral chimeric-gene, and therefore, a multi-resistant ratio was undesirable. In this study, in order to acquire dual-viral resistance, we selected four 300 bp sequences from the 3′ end of the CP genes and the suppressor genes of CMV and PVY^N as target sequences to construct different hpRNA constructions of a chimeric gene. The results show that the transgenic tobacco plants containing each vector exhibited resistance against both the viruses. Constructions targeting the CP genes triggered a greater silencing efficiency. We inferred that different functional genes could affect the gene silencing efficiency (Chen *et al*. 2010). Moreover, with the same target genes, the silencing efficiency of the double-hairpin construction was 50 % greater than that of the single-hairpin construction. The results suggest that the chimeric-gene construction strategy may have more extensive applications.

With the same target genes, there was a significant difference in the silencing efficiency between the two constructs. This difference may be attributed to the efficiency of siRNA processing. The results of the transcript assay show that the siRNA processing efficiency of the double-hairpin constructs with the double-hairpin was significantly greater than that of the single-hairpin constructs. Several studies have indicated that the minimum free energy (ΔG) of the construct may significantly influence the silencing efficiency (Khvorova *et al*. 2003, Schwarz *et al*. 2003). In our study, $\Delta G_{(pHC2bDH)}$ = -1889.49 kJ mol^{-1} was greater than $\Delta G_{(pHC2bSH)}$ = -2445.79 kJ mol^{-1}, and $\Delta G_{(pDCPDH)}$ = -1939.31 kJ mol^{-1} was greater than $\Delta G_{(pDCPSH)}$ = -2478.02 kJ mol^{-1} (ΔG calculated using the formula from the website: http://www.bioinfo.rpi.edu/applications /mfold) which suggests that the double-hairpin was a comparatively steady structure. Duan *et al.* (2008) inferred that target recognition in a highly structured area inhibits the siRNA processing efficiency by influencing siRISC catalysis. In addition, the accessibility of the target site may be another cause of this discrepancy. Previous studies have found that the secondary construction can influence the accessibility of siRNA target sites, affecting the assembly between siRNA and target RNA. In particular, the accessibility of the target

site may be restricted when the siRNA target sites are located in the complex stem-loop structure (Molnár *et al.* 2005, Overhoff *et al*. 2005), thereby reducing the production of siRNA.

In the same type of constructs, construct targeting the CP genes induced a greater silencing efficiency. So we inferred that different functions of gene products working in the viral reproduction may be the cause of this phenomenon. In the PVY genome, the function of the target gene significantly influences the silencing efficiency (Chen *et al*. 2010). Some mRNA binding proteins may block the access of the special siRNAs to the target mRNAs (Qi *et al*. 2009). In addition, the PVY HC protein and the CMV 2b protein, as viral suppressors of RNA silencing, may regulate the production of siRNAs in tobacco (Mallory *et al.* 2002). Moreover, Duan *et al*. (2008) found that although the resistance may be obtained by the selection of optimal accessible target sites in the 3′ end of the CMV 2b gene, the CMV CP gene is a better site to identify siRISC than is the CMV 2b gene.

In transgenic research, T_0 plants are generally heterozygotes. The self-pollinated off-springs of T_0 transgenic plants that show one hybridisation band in the Southern blot analysis should exhibit the 3:1 segregation in kanamycin selection. Our results reveal that most of the tested lines followed a 3:1 ratio, whereas the T_1-pDCPSH-28R line had a 15:1 ratio. We speculate that this phenomenon occurred because of total DNA was restricted into two fragments of a similar length that made them inseparable by electrophoresis. After the inoculation with CMV and PVY^N, the viral disease symptoms were observed only in a small number of the transgenic plants with the kanamycin resistance (the resistance ratio of each line was greater than 5 %). Methylation of promoters in the transgenic plants during the germination and growing periods could account for the reduced expression of the foreign gene, thereby causing susceptibility to viruses (English *et al*. 1996, Guo *et al*., 1999).

References

Audy, P., Palukaitis, P., Slack, S.A., Zaitlin, M.: Replicase-mediated resistance to potato virus Y in transgenic tobacco plants. - Mol. Plant-Microbe. Interact. **7**:15-22, 1994.

Arif, M., Azhar, U., Arshad, M., Zafar, Y., Mansoor, S., Asad, S.: Engineering broad-spectrum resistance against RNA viruses in potato. - Transgenic. Res. **2**:303-11, 2012.

Boucher, 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.

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., Lao, K.Q., Livak, K.J., Guegler, K.J.: Real-time quantification of microRNAs by stem-loop RT-PCR. - Nucl. Acids Res. **33** (Suppl.): e179, 2005.

Chen, X.M., Liu, J., Li Xu., Jiang, F., Xie, X.Y., Zhu, C.X., Wen, F.J.: Inhibiting virus infection by RNA interference of the eight functional genes of the *Potato Virus Y* genome. - J. Phytopathol. **158**: 776-784, 2010.

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.

English, J.J., Mueller, E. and Baulcombe, D.C.: Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear gene. - Plant Cell **8**: 179-188, 1996.

Guo, H.S., López-Moya, J.J., García, J.A.: Mitotic stability of infection-induced resistance to *Plum Pox Potyvirus* associated with transgene silencing and DNA methylation. - Mol. Plant-Microbe. Interact. **12**: 103-111, 1999.

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, 2001 [In Chinese].

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J.: An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. - Nature **404**: 293-296, 2000.

Hannon, G.J.: RNA interference. - Nature **418**: 244-251, 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.

Huang, J., Ge, X., Sun, M.: Modified CTAB protocol using a silica matrix for isolation of plant genomic DNA. - Biotechnique **28**: 432-434, 2000.

Jan, F.J., Fagoaga, C., Pang, S.Z., Gonsalves, D.: A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. - J. gen. Virol. **81**: 2103-2109, 2000.

Khvorova, A., Reynolds, A., Jayasena, S.D.: Functional siRNAs and miRNAs exhibit strand bias. - Cell **115**: 209-216, 2003.

Kotlizky, G., Katz, A., Van Der Laak, J., Boyko, V., Lapidot, M., Beachy, R.N., Heinlein, M., Epel, B.L.: A disfunctional movement protein of *Tobacco mosaic virus* interferes with targeting of wild-type movement protein to microtubules. - Mol. Plant-Microbe. Interact. **14**:895-904, 2001.

Kung, Y.J., Bau, H.J., Wu, Y.L., Huang, C.H., Chen, T.M., Yeh, S.D.: Generation of transgenic papaya with double resistance to *Papaya ringspot virus* and *Papaya leaf-distortion mosaic virus*. - Phytopathology **99**: 1312-1320, 2009.

Lin, C.Y., Ku, H.M., Tsai, W.S., Jan, F.J.: Resistance to a DNA and a RNA virus in transgenic plants by using a single chimeric transgene construct. - Transgenic. Res. **20**: 261-270, 2011.

Mallory, A.C., Reinhart, B.J., Bartel, D., Vance, V.B., Bowman, M.H.: From the cover: a viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. - Proc. nat. Acad. Sci. USA **99**: 15228-15233, 2002.

Molnár, A., Csorba, T., Lakatos, L., Várallyay, E., Lacomme, C., Burgyán, J.: Plant virus-derived small interfering RNAs

originate predominantly from highly structured single-stranded viral RNAs. - J. Virol. **79**: 7812-7818, 2005.

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.

Praveen, S., Mangrauthia, S.K., Singh, P., Mishra, A.K.: Behavior of RNAi suppressor protein 2b of *Cucumber mosaic virus in planta* in presence and absence of virus. - Virus Genes **37**: 96-102, 2008.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., Tepfer, M.: Strategies for antiviral resistance in transgenic plants. - Mol. Plant. Pathol. **9**: 73-83, 2008.

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.

Schwarz, D.S., Hutvágner, G., Du, T.T., Xu, Z.S., Aronin, N., Zamore, P.D.: Asymmetry in the assembly of the RNAi enzyme complex. - Cell **115**: 199-208, 2003.

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

Takeshita, M., Suzuki, M., Takanami, Y.: Combination of amino acids in the 3a protein and the coat protein of *Cucumber mosaic virus* determine symptom expression and viral spread in bottle gourd. - Arch. Virol. **146**: 697-711, 2001.

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

Watanabe, Y.: Overview of plant RNAi. - Methods mol. Biol. **744**: 1-11, 2011.

Wu, H.W., Yu, T.A., Raja, J.A.J., Christopher, S.J., Wang, S.L., Yeh, S.D.: Double-virus resistance of transgenic oriental melon conferred by untranslatable chimeric construct carrying partial coat protein genes of two viruses. - Plant. Dis. **94**: 1341-1347, 2010.

Yoshinari, K., Makoto, M., Kazunari, T.: Effects on RNAi of the tight structure, sequence and position of the target region. - Nucl. Acids Res. **32**: 691-699, 2004.

Zhang, C., Song, Y., Jiang, F., Li, G., Jiang, Y., Zhu, C., Wen, F.: Virus resistance obtained in transgenic tobacco and rice by RNA interference using promoters with distinct activity. - Biol. Plant. **56**: 742-748, 2012.

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.