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Virus resistance obtained in transgenic tobacco and rice by RNA interference using promoters with distinct activity

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Abstract

To induce virus resistance in tobacco and rice we constructed hairpin RNA expression system harbouring inverted repeat fragments of coat protein cDNA of *Potato virus Y* (PVY) or *Rice stripe virus* (RSV). These structures were driven by three promoters [cauliflower mosaic virus 35S (CaMV 35S), polyubiqutin gene of maize (Ubi), and *Pharbitis nil* leucine zipper gene (PNZIP)] which have different tissue-specific activity. PVY resistance ratios were 65.18, 24.33 and 83.54 % in transgenic tobacco plants harboring p35S-PVY, pUbi-PVY and pPNZIP-PVY. RSV resistance was 16.21, 28.61 and 29.33 % in transgenic rice plants harboring p35S-RSV, pUbi-RSV and pPNZIP-RSV. Northern blotting and GUS assay demonstrated that virus resistance levels were related to promoter activity. Therefore, choice of the more effective and tissue-specific promoter to reinforce transcription of hpRNAs will favour the cultivation of highly virus-resistant transgenic plants.

Additional key words: *Agrobacterium tumefaciens, Nicotiana tabacum*, *Oryza sativa*, *Potato virus Y*, *Rice stripe virus*.

Introduction

Potato virus Y (PVY) belongs to the genus *Potyvirus* of the family *Potyviridae*, a positive-sense single-stranded RNA genome that can infect more than 120 kinds of plants from *Solanaceae, Amaranthaceae, Chenopodiaceae, Compsitae, Leguminosae*, *etc*. *Rice stripe virus* (RSV) is a representation of *Tenuivirus*, which is composed of four single-stranded RNA genomes that can infect over 37 kinds of plants such as rice, maize, wheat, oat and chestnut (Toriyama 1986). For the past few years in China, outbreaks of PVY and RSV on tobacco and rice occurred epidemically, causing a considerable reduction in tobacco and rice production.

The cultivation of resistant cultivars is considered the most economic and effective way of controlling viral disease, however, virus-resistant genes in plants is few (Pushpalatha *et al.* 2011). The pathogen-derived resistance through introducing various viral sequences in plants provides a new approach for cultivating transgenic virus-resistant plants. It has been validated that the novel nature of this resistance is RNA interference (RNAi; Prins and Goldbach 1996). RNAi is an umbrella term to describe homology-dependent gene silencing. It is triggered by endogenous or exogenous double stranded RNA (dsRNA) which will be cleaved into 21 - 24 nucleotides small interfering RNA (siRNA) by endonuclease Dicer. The siRNA is then incorporated into the RNA-induced silencing complex (RISC) and acts as a guide for recognizing and degrading specific complementary RNA, so that exogenous genetic factors, such as viruses, transposons, and transgenes are rejected (Deleris *et al.* 2006). Additionally, siRNA combines with target RNA to synthesize more dsRNAs by RNA-dependent RNA

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Abbreviations: CaMV 35S - cauliflower mosaic virus 35S; CP - coat protein; dsRNA - double-stranded RNA; GUS - β-glucuronidase; hpRNA - hairpin RNA; PNZIP - *Pharbitis nil* leucine zipper gene; PTGS - post-transcriptional gene silencing; PVY - *Potato virus Y*; RdRP - RNA-dependent RNA polymerase; RISC - RNA-induced silencing complex; RMGS - RNA-mediated gene silencing; RNAi - RNA interference; RSV - *Rice stripe virus*; siRNA - small interfering RNA; SSC - sodium chloride/sodium citrate; Ubi - maize polyubiqutin gene; vcRNA3 - viral complementary RNA3.
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polymerase (RdRP). In plants, this process is called post-transcriptional gene silencing (PTGS; Cogoni and Macino 1997).

The *Cauliflower mosaic virus* (CaMV) 35S promoter is a constitutive promoter, widely used in gene transcription especially of dicotyledons. The polyubiqutin gene of maize (Ubi) is a strong constitutive promoter used in monocotyledons. The *Pharbitis nil* leucine zipper gene (PNZIP) promoter, which generates strong expression in photosynthetic tissues, has been used in both dicotyledons and monocotyledons. William *et al.* (1992) found that using stronger promoters usually induced generous gene expression. Previous investigations validated that the promoter activity influenced the efficiency of RNA-mediated gene silencing (RMGS; Que *et al.* 1997, Nakatsuka *et al.* 2007, Wang *et al.* 2008). To date, no systematic investigation of the effect of promoters on RNAi-mediated virus resistance was published. In this research, plant expression vectors harboring hairpin RNA (hpRNA) structure targeting PVY coat protein (*CP*) gene and RSV *CP* were constructed and transformed into tobacco and rice. The effects of the CaMV35S, Ubi, and PNZIP promoter activities on RNAi-conferred virus resistance in transgenic tobacco and rice plants were investigated.

Materials and methods

The cDNA fragments (500 and 400 bp) were amplified from 3' ends of PVY *CP* gene (GenBank acc. No. GU550507) and RSV *CP* gene (GenBank acc. No. DQ108406) by designed specific primers (Table 1). Then these cDNA fragments were inserted reversely into binary vectors between the restriction enzymes sites *Bam*HI and *Kpn*I, *Kpn*I and *Sac*I, respectively. Six hairpin figure RNAi expression vectors (p35S-PVY, pUbi-PVY and pPNZIP-PVY as well as p35S-RSV, pUbi-RSV and pPNZIP-RSV constructs) were obtained (Fig. 1*A*,*C*). The report vectors which expressed β-glucuronidase (*gus*) gene (p35S-gus-npt, pUbi-gus-npt, pPNZIP-gus-npt, p35S-gus-hph, pUbi-gus-hph and pPNZIP-gus-hph) were obtained by inserting the *gus* gene into three kinds of recombinant express vectors driven by different promoters between the *Bam*HI and *Kpn*I sites (Fig. 1*B*,*D*).

Table 1. Description of oligonucleotide primers used in this study. They are for amplification of the *gus* gene fragment and the sense and antisense fragments (500 and 400 bp) corresponding to the 3′ ends of the full-length PVY-CP and RSV-CP gene to construct the hairpin structure. Underlined sequences denote restriction endonuclease cleavage sites.

Fragment	Primer	Restriction enzyme
GUS-5′	GCGCGGATCCATGTTACGTCCTGTAGAAAC	*Bam*HI
GUS-3′	GCGCGGTACCAGCTCGGTAGCAATTCCC	*Kpn*I
PVYCP500-5′	GCGCGGATCCGACATAGGAGAAACTGAAA	*Bam*HI
PVYCP500-3′	GCGCGGTACCCATGTTCTTCACTCCAAGTA	*Kpn*I
PVYCP400-5′	GCGCGAGCTCGACATAGGAGAAACTGAAAT	*Sac*I
PVYCP400-3′	GCGCGGTACCCCGAAAAGTCGAGATTGAG	*Kpn*I
RSVCP500-5′	GCGCGGATCCCTTACTGTGGGACTATGTTC	*Bam*HI
RSVCP500-3′	GCGCGGTACCCTAGTCATCTGCACCTTCTG	*Kpn*I
RSVCP400-5′	GCGCGAGCTCCTTACTGTGGGACTATGTTC	*Sac*I
RSVCP400-3′	GCGCGGTACCGTTTGCTCTGTTGAGCCAAG	*Kpn*I

The recombinant binary vectors p35S-PVY, pUbi-PVY, pPNZIP-PVY, p35S-gus-npt, pUbi-gus-npt and pPNZIP-gus-npt were transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thawing method (Rhodes *et al.* 1988), and eventually introduced into tobacco (*Nicotiana tabacum* L.) cv. NC89 *via* leaf disk transformation (Horsch *et al.* 1985). Moreover, recombinant binary vectors p35S-RSV, pUbi-RSV, pPNZIP-RSV, p35S-gus-hph, pUbi-gus-hph and pPNZIP-gus-hph were transferred into *A. tumefaciens* strain EHA105 by the electroporation method (Taketo 1988), and introduced into rice (*Oryza sativa* L. cv. Zhonghua 11) according to modified protocol by Hiei *et al.* (1994). T_0 transformants were detected by PCR, and PCR-positive plants were allowed to self-pollinate. T_1 plants were obtained by screening at 100 mg dm^{-3} kanamycin or hygromycin B.

The detection of GUS activity was conducted according to Bradford (1976) and Jefferson and Bevan (1987). 20 plants were selected in each transgenic group and wild-type of tobacco or rice. GUS activity was denoted as μmol(4-methylumbelliferone) mg^{-1}(protein) min^{-1}. When T_1 generation of tobacco plants reached the 4 to 5 leaf stage, two upper leaves were infected with PVY^N which was diluted in phosphate buffer and saline buffer at the 1:1 ratio. When T_1 rice seedlings reached the 5 to 10 leaf stage, more than 20 seedlings were selected for inoculation with four instars H population of *Laodelphgax striatellus* per rice. These infection assays were repeated three times. Symptoms of disease were

observed and recorded each day for 4 weeks after inoculation. Virus content was determined by indirect ELISA methods (Guo *et al.* 2001). The resistance ratio of RSV was represented by the infection rate (percentage of infected plants in total number of tested plants) and resistance ratio (infection rate of wild-type plants - infection rate of transgenic plants). The data analysis was carried out by software *SPSS* (*v. 19*).

Total RNA was extracted by *Trizol* reagent (*Invitrogen*, Carlsbad, USA); 20 μg total RNA was electrophoresed on a 1.2 % agarose gel containing 1 % formaldehyde, and transferred to *Hybond*^{TM}-N^{+} membranes (*Amersham*, Chalfont, UK) with 20× SSC (Sambrook *et al.* 2001). SiRNAs were extracted with the *PureLink*^{TM} miRNA isolation kit (*Invitrogen*) according to the manufacturer's instructions. The samples were loaded onto a 15 % polyacrylamide gel containing 7 M urea, electro-transferred, and fixed by UV cross-linking. Northern blot hybridization and DIG-labeled RNA probe were conducted by transcribing the target regions according to the manufacturer's instructions (*DIG* Northern starter kit*, Roche*, Mannheim, Germany).

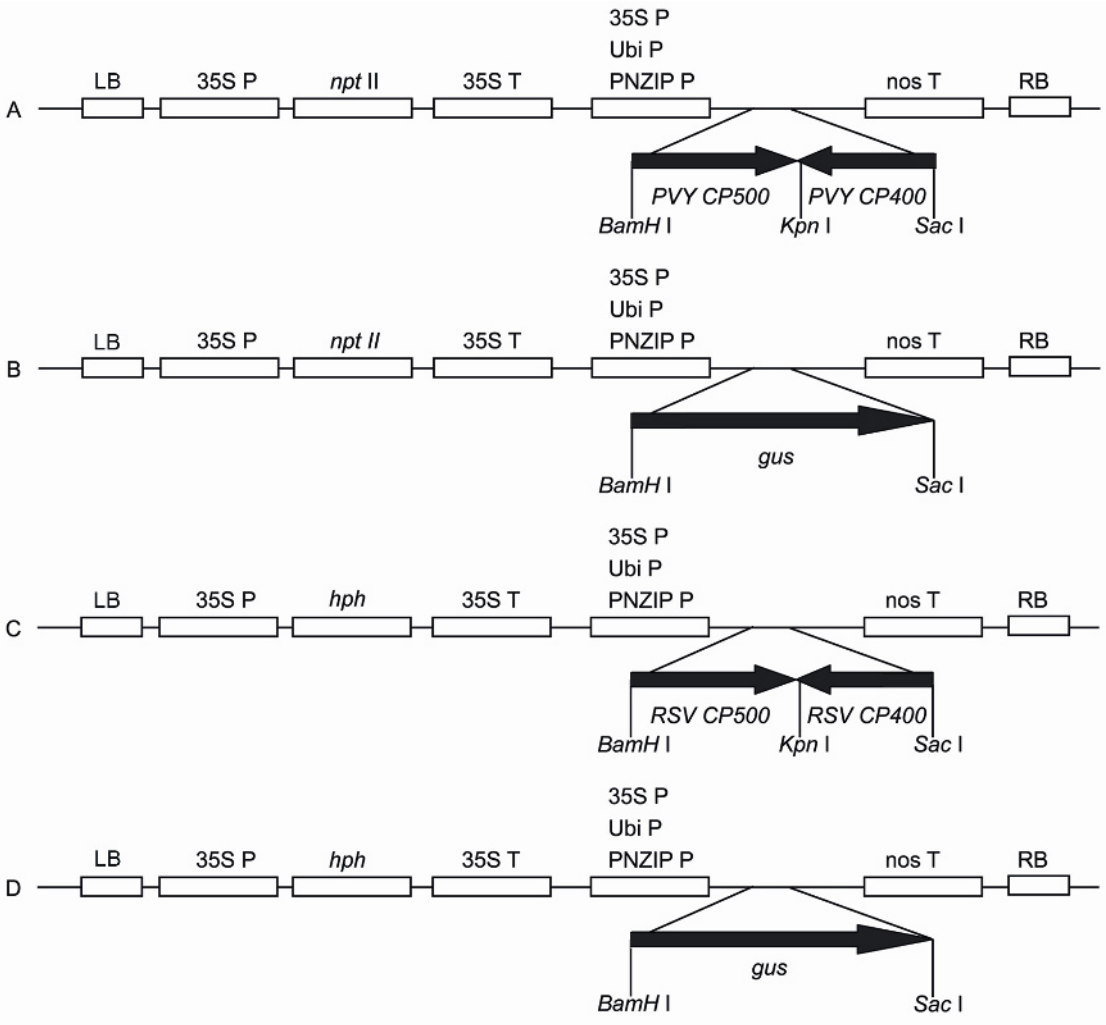

Fig. 1. Construction of binary vectors. Binary vectors for tobacco transformation (*A* - including *npt*II resistance gene and reverse repeat sequence of PVY *CP* gene, *B* - including *npt*II resistance gene and *gus* gene). Binary vectors for rice transformation (*C* - including *hph* gene and reverse repeat sequence of RSV *CP* gene, *D* - including *hph* resistance gene and *gus* gene). RB - right border of T-DNA; 35S P - CaMV 35S promoter; Ubi P - the polyubiquitin gene of maize promoter; PNZIP P - the *Pharbitis nil* leucine zipper gene promoter; *npt*II - neomycin phosphotransferase II gene; *hph* - hygromycin B phosphotransferase gene; 35S T - 35S terminator; nos T - nopaline synthase (Nos) terminator; LB - left border of T-DNA; restriction sites - *Bam*HI, *Kpn*I, *Sac*I.

Results

To evaluate the strength of each promoter in transgenic tobacco and rice, we constructed six vectors (p35S-gus-npt, pUbi-gus-npt, pPNZIP-gus-npt, p35S-gus-hph, pUbi-gus-hph and pPNZIP-gus-hph)

harboring *gus* gene driven under the control of the three different promoters (Fig. 1*B*,*D*) and transformed into tobacco and rice plants, respectively. GUS activity was remarkably different among the three promoters (Table 2). The transgenic tobacco carrying pPNZIP-gus-npt had the highest expression, which was 9-fold higher than that carrying p35S-gus-npt. The Ubi promoter showed much lower activity, only 0.5-fold higher compared with 35S promoter. In transgenic rice, both PNZIP and Ubi promoter had 4 - 5 fold higher activity compared with 35S promoter.

In order to investigate the effect on RNAi silencing efficiency caused by different promoters, six inverted repeat recombinant binary vectors targeting PVY *CP* gene or RSV *CP* gene (p35S-PVY, pUbi-PVY, pPNZIP-PVY p35S-RSV, pUbi-RSV and pPNZIP-RSV) were constructed (Fig. 1*A*,*C*). Then they were transformed into tobacco cv. NC89 and rice cv. Zhonghua 11 by *A. tumefaciens*. After transformation and self-pollination, 112, 115 and 152 tobacco plants were obtained using p35S-PVY, pUbi-PVY, pPNZIP-PVY, 83, 68 and 75 rice plants were obtained using p35S-RSV, pUbi-RSV, pPNZIP-RSV, respectively. These T_1 plants were used in subsequent virus resistance tests.

Table 2. GUS activity [μmol(4-methylumbelliferone) mg^{-1} (soluble protein) min^{-1}] in transgenic tobacco and rice plants (*gus* gene was driven by different promoters. WT - wild type plants. Means ± SE, $n = 20$.

Promoter	Tobacco	Rice
PNZIP	55.01 ± 0.92	25.02 ± 0.83
Ubi	3.22 ± 0.38	24.65 ± 0.48
35S	6.11 ± 0.47	5.12 ± 0.25
WT	0.16 ± 0.01	0.11 ± 0.01

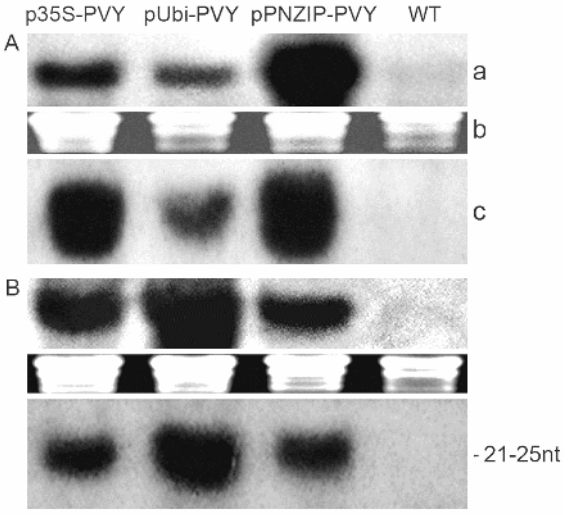

Fig. 2. Expression analysis of RNAi constructs. Northern blot analysis was performed to determine the accumulation of transcripts before virus inoculation. *A* - the accumulation of transcripts and siRNAs in transgenic plants containing p35S-PVY, pUbi-PVY, pPNZIP-PVY. *B* - the accumulation of transcripts and siRNAs in the transgenic plants containing p35S-RSV, pUbi-RSV, pPNZIP-RSV. (a - accumulation of transcripts, b - ethidium bromide-stained ribosomal RNA was used to show that an equal amount of total RNA was loaded, c - siRNAs in the transgenic plants using the same probes).

Table 3. Responses of transgenic and nontransgenic plants to PVY^N infection. Resistance analysis of transgenic plants challenged with PVY^N through symptoms monitoring daily from 1 to 4 weeks after inoculation. Means ± SE, $n = 3$

Transgene	Total number of plants tested	Number of resistant plants	Resistance ratio [%]
p35S-PVY	112	73	65.18 ± 1.81
pUbi-PVY	115	28	24.33 ± 0.66
pPNZIP-PVY	152	126	83.54 ± 1.37
WT (NC89)	70	0	0

To examine the virus resistance conferred by hpRNAi structure driving under the control of three different promoters, a large number of transgenic plants were screened. Wild-type NC89 and Zhonghua11 served as the negative control. In transgenic tobacco, some plants were resistant, showing no susceptibility symptoms in the entire lifetime and the PVY content did not significantly differ from that of uninfected plant. The others were susceptible showing symptoms similar to that of inoculated-wild type, and PVY content was higher than in uninfected plant. The statistical results showed that the ratios of resistant transgenic tobacco plants were 65.18, 24.33 and 83.54 % using constructs p35S-PVY, pUbi-PVY and pPNZIP-PVY, respectively (Table 3). To reduce the error caused by the inoculation of *Laodelphgax striatellus,* the RRR was calculated to represent the resistance level to RSV. The results of rice resistance analysis showed that the numbers of susceptible plants in transgenic lines were less than that in control Zhonghua 11. The relative ratios of resistant transgenic rice were 16.21, 28.61 and 29.33 % in plants using p35S-RSV, pUbi-RSV and pPNZIP-RSV constructs, respectively (Table 4). In conclusion, in our study, the PNZIP promoter was more effective in driving the RNAi system to confer virus resistance in transgenic tobacco and rice. We also tested the expression level of RNAi silencing structures under the control of the three promoters. The expression analysis was performed in each transgenic line before the virus was inoculated. Northern blot showed that the dsRNA transcripts were detected in all the transgenic plants, however, the accumulations of transcript were discrepant in different transgenic plants. The accumulation of transcript in pPNZIP-PVY tobacco plant was more than those in p35S-PVY and pUbi-PVY tobacco plants, while p35S-RSV rice plant was less than pUbi-RSV and pPNZIP-RSV rice plants (Fig. 2*A*,*B*). In the subsequent

Table 4. Responses of transgenic and nontransgenic plants to RSV infection. Resistance analysis of transgenic plants challenged with RSV through symptoms monitoring daily from 1 to 4 weeks after inoculation. Means ± SE, $n = 3$.

Transgene	Total number of plant tested	Number of resistant plants	Infection ratio [%]	Resistance ratio [%]
p35S-RSV	83	30	63.79 ± 0.24	16.21 ± 0.59
pUbi-RSV	68	34	51.39 ± 0.04	28.61 ± 0.39
pPNZIP-RSV	75	37	50.67 ± 0.17	29.33 ± 0.52
WT (Zhonghua 11)	75	15	80.00 ± 0.35	-

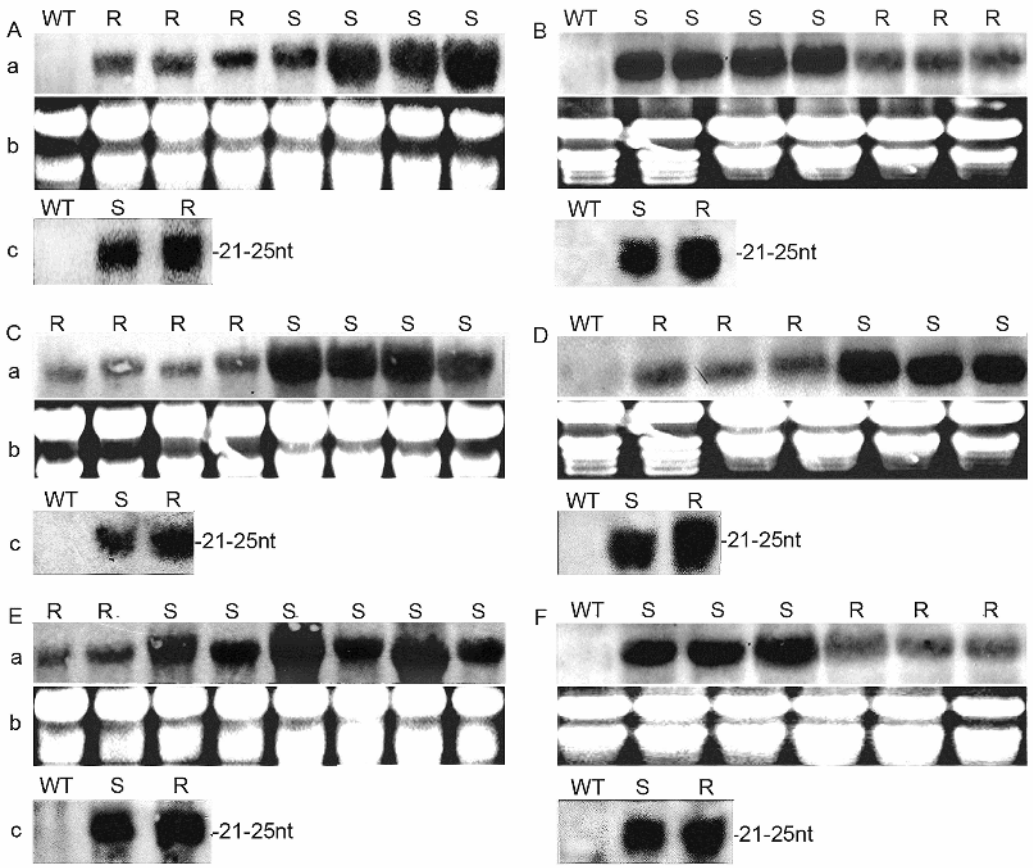

Fig. 3. Northern blot analysis of the accumulation of transcripts and siRNAs in virus-inoculated transgenic plants. Total RNA and siRNAs were extracted from different transgenic plants containing p35S-PVY (*A*), p35S-RSV (*B*), pUbi-PVY (*C*), pUbi-RSV (*D*), pPNZIP-PVY (*E*) and pPNZIP-RSV (*F*) (*a* - results of Northern blot, *b* - rRNA was used to show that an equal amount of total RNA was loaded, c - result of Northern blot of siRNAs, WT - wild type plants; S - susceptible plants; R - resistant plants).

Northern blot of siRNAs, we detected siRNAs in all transgenic tobacco/rice plants, while they were undetectable in wild-type plants (Fig. 2*A*,*B*). Additionally, the accumulation of siRNAs corresponded with the accumulation of the hpRNA transcripts, and there was a line-correlation between the expression of siRNAs and the accumulation of the transcripts. These results indicated that the choice of different promoter affected the expression efficiency of the RNAi constructs, and hairpin transcripts could be recognized effectively to generate specific siRNAs by PTGS system of host plants.

To investigate if there is a relationship between transcript accumulation and virus resistance, total RNAs were extracted from virus inoculated plants. Resistant and susceptible plants were stochastic selected from each transgenic group. Northern blot showed that there was no hybridization signal in wild-type tobacco and rice, while the transgenic tobacco and rice showed specific hybridization bands, and the accumulation of transcripts RNA in resistant plants was far less than that in the susceptible plants (Fig. 3*A*-*F*). Further siRNA analysis showed that hybridization signals were detected in all

transgenic tobacco or rice plants and the accumulation of siRNAs in susceptible plants were as much as in resistant plants, but no signal was observed in wild-type plants (Fig. 3*A-F*). All these results demonstrated that the virus resistance was indeed mediated by RNA, however, there was no obvious correlation between the expression level of sequence-specific siRNAs and virus resistance.

Discussion

In this study, we evaluated the effect of promoters on hpRNA-mediated antivirus test in dicotyledons and monocotyledons. The CaMV35S, Ubi, and PNZIP promoters were chosen for the investigation of the expression of RNA-mediated virus resistance in transgenic tobacco and rice. The results demonstrate that promoter activity significantly affects transformation efficiency; high gene expression may induce high levels of virus resistance. The RNAi constructs with PNZIP promoter could induce the most efficient transformation.

In our previous experiments, hpRNA or ihpRNA constructs were transformed into plants. hpRNA structure could improve the efficiency of gene silencing, but 20 - 40 % of the transgenic plants showed no gene silencing. These results indicated that some other factors than gene structure affected the efficiency of gene silencing. In an earlier report, Nakatsuka (2007) and Wang *et al.* (2008) showed that the suppression efficiency of reporter genes or target genes corresponded with respective promoter activity. In our research, we also demonstrated that RNA-mediated virus resistance could be controlled by promoter and the transcriptional efficiency of respective genes. The expression and accumulation of a certain level of dsRNA (belong to aberrant RNA) to reach a "threshold" was indispensable (Que *et al.* 1997), these dsRNAs would be diced into the siRNAs to confer virus resistance. The use of effective promoter led to accumulation of more transcripts and so to production of more primary siRNAs. Our result supported the view that the pre-existence of a certain number of virus-specific siRNAs in plants seemed to be a crucial determinant factor for obtaining virus resistance (Abhary *et al.* 2006).

When we detected the accumulation of specific siRNAs in the virus infected transgenic plants, we found that the accumulation of specific siRNAs in resistant plants was not higher than in susceptible plants. One possible explanation is, that siRNAs are target-accessible and not equally distributed in transgenic plants, although the total siRNAs content is similar (Jiang *et al*. 2011). The other explanation is that the accumulation of siRNAs might not be the only determinant of susceptibility to virus infection.

In our results, RSV CP-mediated virus resistance in rice was much lower compared with PVY CP-mediated virus resistance. This diversity may be due to several reasons: *1*) the differences between the plant species as tobacco is dicotyledonous, but rice is monocotyledonous; *2*) the differences between gene expression (GUS activity in tobacco was higher than in rice); and *3*) the differences between the viruses. The PVY genome is a single-stranded positive-sense RNA, and contains a single, large open reading frame encoding a large polyprotein. The *CP* gene locates the 3′ end of the genome (Urcuqui-Inchima *et al.* 2001). Some studies showed that the gene silencing signal extended along the 5′ - 3′ direction (Braunstein *et al.* 2002). Therefore, the entire genome of PVY can be seen as the target of gene silencing. However, the RSV is composed of multiple genomes, including the drug justice complementary strand RNA3 (viral complementary RNA3, vcRNA3) encoding the *CP* gene. The RNA1, RNA2, and RNA4 are not direct targets of *CP* gene siRNA, and siRNAs derived from these nontarget RNAs rapidly accumulate in plant cells, which may lead to the saturation of siRISC and impair *CP* gene siRNA antiviral efficacy. It may also be possible that the *CP* gene target site is not in the optimal siRISC-target recognizing region. A similar phenomenon has been found in an amiR2b-mediated CMV resistance study (Duan *et al.* 2008).

Based on all our results, we propose that the RNAi construct under PNZIP promoter could induce efficient virus resistance in transgenic dicotyledons and monocotyledons. These results provide the foundation for future cultivation of highly virus-resistant transgenic plants using RNAi-mediated virus resistance.

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