Production of marker-free and RSV-resistant transgenic rice using a twin T-DNA system and RNAi

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A twin T-DNA system is a convenient strategy for creating selectable marker-free transgenic plants. The standard transformation plasmid, pCAMBIA 1300, was modified into a binary vector consisting of two separate T-DNAs, one of which contained the hygromycin phosphotransferase (*hpt*) marker gene. Using this binary vector, we constructed two vectors that expressed inverted-repeat (IR) structures targeting the rice stripe virus (RSV) coat protein (CP) gene and the special-disease protein (SP) gene. Transgenic rice lines were obtained via *Agrobacterium*-mediated transformation. Seven independent clones harbouring both the *hpt* marker gene and the target genes (RSV CP or SP) were obtained in the primary transformants of pDTRSVCP and pDTRSVSP, respectively. The segregation frequencies of the target gene and the marker gene in the T_1 plants were 8.72% for pDTRSVCP and 12.33% for pDTRSVSP. Two of the pDTRSVCP lines and three pDTRSVSP lines harbouring the homozygous target gene, but not the *hpt* gene, were strongly resistant to RSV. A molecular analysis of the resistant transgenic plants confirmed the stable integration and expression of the target genes. The resistant transgenic plants displayed lower levels of the transgene transcripts and specific small interfering RNAs, suggesting that RNAi induced the viral resistance.

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1. Introduction

Transgenic crops are currently grown in 28 countries, and the total cultivated area of transgenic crops was estimated to reach 170.3 million hectares in 2012 (James 2012). Selection marker genes that encode resistance to antibiotics and herbicides are widely used to identify the rare transformation of crop plants in which the foreign DNA has integrated (Sundar and Sakthivel 2008); aside from this function, these genes are non-essential. Over the past decade, the use of genes that promote resistance to antibiotics and herbicides has raised vast public concern due to their potential risk to ecology, food safety and potentially significant interactions between these transgenic traits and the environment (Kuiper *et al.* 2001; Dale *et al.* 2002; Tuteja *et al.* 2012). Therefore, studies regarding the elimination of marker genes after use have gained increasing interest, leading to several strategies. These strategies include cotransformation (Breitler *et al.* 2004; Wakasa *et al.* 2012), transposition (Goldsbrough *et al.* 1993; Cotsaftis *et al.* 2002), homologous recombination (Maliga, 2002) and site-specific recombination (Dale and Ow 1991; Gleave *et al.* 1999; Puchta 2000). Cotransformation of the desired gene and the marker gene, which are supplied by two T-DNAs located within the same replicon, is considered a convenient strategy for producing marker-free transgenic crops (Puchta 2003). In a twin T-DNAs system, one of the T-DNAs contains the gene of interest and the other carries a marker gene cassette. Selection marker genes can subsequently segregate from genes of interest in the progeny during sexual reproduction (Komari *et al.* 1996).

Keywords. Marker-free; rice stripe virus; RNA interference; transgenic rice; twin T-DNA

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Rice (*Oryza. sativa L.*) is an important crop that serves as staple food for approximately half of the world's population. It is susceptible to several viral pathogens, such as the rice stripe virus (RSV), which is one of the most important threats to rice in East Asia, negatively effecting worldwide crop production on an annual basis (Wei *et al.* 2009). The RSV genome consists of four single-stranded RNA segments, designated as RNAs 1 to 4. RNA 1 has negative polarity, whereas the other segments use an ambisense coding strategy. Molecular virology studies have decoded each of the seven proteins encoded by the RSV genome segments. The complementary-sense RNA 3 encodes the coat protein (CP), and RNA 4 encodes the special-disease protein (SP). These two proteins accumulate in infected plants, and they are closely correlated with the severity of chlorosis and mosaic (Toriyama 1986; Zhu *et al.* 1991).

Strategies based on the concept of pathogen-derived resistance (PDR) are considered to be the most powerful approaches for cultivating virus-resistant crops and conferring viral resistance in plants to combat infections (Sanford and Johnson 1985; Simón-Mateo and García 2011). Previous studies revealed the mechanism of RNA-mediated virus resistance (RMVR), known as post-transcriptional gene silencing (PTGS), which represents a potential strategy with broad applications and practical significance in anti-virus genetic engineering (Mohanpuria *et al.* 2008; Llave 2010).

In the present study, we constructed twin T-DNA vectors that separately target the 400 nucleotides of RSV CP and SP. Through *Agrobacterium*-mediated transformation, we obtained selectable, marker-free transgenic rice with strong RSV resistance to develop marker-free and RSV-resistant rice varieties.

2. Materials and methods

2.1 *Construction of plasmids*

Primers were designed to obtain a *Sac*I-*Eco*RI segment (996 bp) of plasmid pBI121 (Chen *et al.* 2003) containing the nopaline synthase (NOS) terminator left border–right border fragment. A *Hin*dIII-*Pst*I segment containing the maize (*Zea mays*) ubiquitin (Ubi) promoter was amplified via PCR using the primer pairs shown in table 1. Then we modified the standard transformation plasmid pCAMBIA 1300 (Hajdukiewicz *et al.* 1994) by inserting the *Sac*I-*Eco*RI segment and the *Hin*dIII-*Pst*I segment. IR constructs containing a phospholipase D intron (accession number AB001919) as the spacer for dsRNA transcription were inserted between the *Pst*I and the *Sac*I sites of the resultant plasmids. T-DNA one contained an *hpt* cassette, and T-DNA two carried an IR region that targets the 400-nucleotide middle regions of either the RSV CP or the SP genome segment. The final two T-DNA binary vectors were designated as pDTRSVCP and pDTRSVSP (figure 1). The stem regions of the IR constructs were amplified using either the RSV CP cDNA (accession number DQ108406) or the SP cDNA (accession number EF538684) as the template.

Table 1. Primer sequences for gene amplification

Fragment	Primer sequence		Restriction enzyme
NOS-LB-RB (996bp)	P1-5′	5′-GCGC GAGCTC GATCGTTCAAACATTTGGC-3′	*Sac*I
	P1-3′-1	5′-GCGC GAATTC TGACAGGATATATTGGCGGGT AAAC AGTGGTGATTTTGTGCCG-3′	*EcoR*I
	P1-3′-2	5′-GCGC GAATTC TGACAGGAT ATATTGGCG-3′	*EcoR*I
Ubi (1996bp)	P2-5′	5′-GCGC AAGCTT TGCAGCGTGACCCGGTCG-3′	*Hind*III
	P2-3′	5′-GCGC CTGCAG AAGTAACACCAAACAACAGGG-3′	*Pst* I
RSVCP-1 (400bp)	P3-5′	5′-GCGC GGATCC CTTACTGTGGGACTATGTTC-3′	*Bam*HI
	P3-3′	5′-GCGC CTGCAG GTTTGCTCTGTTGAGCCAAG-3′	*Pst* I
RSVCP-2 (400bp)	P4-5′	5′-GCGC GGTACC CTTACTGTGGGACTATGTTC-3′	*Kpn*I
	P4-3′	5′-GCGC GAGCTC GTTTGCTCTGTTGAGCCAAG-3′	*Sac*I
RSVSP-1 (400bp)	P5-5′	5′-GCGC GGATCC GTCCTATTGTAGGCCTAG-3′	*Bam*HI
	P5-3′	5′-GCGC CTGCAG GCCATCTTGATAATTTGATC-3′	*Pst* I
RSVSP-2 (400bp)	P6-5′	5′-GCGC GGTACC GTCCTATTGTAGGCCTAG-3′	*Kpn*I
	P6-3′	5′-GCGC GAGCTC GCCATCTTGATAATTTGATC-3′	*Sac*I
PLD intron (400bp)	P7-5′	5′-GCGC GGATCC CATTGGAGATCCATAAGAG-3′	*Bam*HI
	P7-3′	5′-GCGC GGTACC TACGCAATTACTTCATTCC-3′	*Kpn*I
Hpt (499bp)	P8-5′	5′- TAGGAGGGCGTGGATATGTC-3′	
	P8-3′	5′- TACACAGCCATCGGTCCAGA-3′	

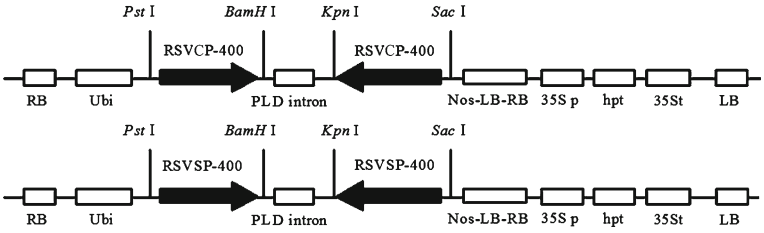

Figure 1. T-DNA regions of pDTRSVCP and pDTRSVSP. RB: Right border; LB: Left border; Ubi: Ubiquitin promoter; RSVCP-400: fragments targeting RSV CP middle section (nt 471-870 of the complete cds); RSVSP-400: fragments targeting RSV SP middle section (nt 38-437 of the complete cds); PLD: Phospholipase D intron of rice; NOS: nopaline synthetase terminator; 35S p: Cauliflower mosaic virus (CaMV) 35S promoter; *hpt*: Hygromycin phosphotransferase; 35S t: CaMV 35S terminator.

2.2 *Transformation of rice calli*

Rice calli (cv. Lindao 10) were transformed using *A. tumefaciens* strain EHA105 based on a previously published method (Toki *et al.* 2006). The transformants were selected in medium containing hygromycin B (50 mg/L). The transgenic plants were subsequently generated from hygromycin-resistant (H-R) calli.

2.3 *Screening by PCR*

We amplified the transgene-specific 400 bp fragments of the CP and the SP segment of RSV and the *hpt* gene (table 2). The PCR cycles used to detect the transgenes were as follows: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 54°C, 45 s at 72°C, and finally 8 min at 72°C. The PCR products were fractionated by electrophoresis on a 1.0% (wt/vol) agarose gel to detect the 400 bp fragments of the target genes and the 500 bp of the *hpt* gene.

2.4 *DNA extraction and Southern blot analysis*

Genomic DNA was extracted from selected transgenic rice lines and a wild-type rice plant using the phenol method (Liu *et al.* 1995). A total of 30 μg of genomic DNA from each sample was digested with *Hin*d III, fractionated via electrophoresis on a 1% agarose gel, and transferred onto a Hybond-N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK). For hybridization, the 400 bp target sequences of the RSV CP and RSV SP genes served as the template for synthesising DIG-labelled probes according to the protocol in the DIG Applications Manual. Southern hybridization was carried out according to the method

Table 2. PCR and leaf painting analysis of T_0 transformation events derived from pDTRSVCP and pDTRSVSP

T_0 plants	H-R	PCR of *hpt*	PCR of CP	T_0 plants	H-R	PCR of *hpt*	PCR of SP
CP-1	+	+	+	SP-1	+	+	−
CP-2	+	+	−	SP-2	+	+	+
CP-3	+	+	+	SP-3	+	+	+
CP-4	+	+	−	SP-4	+	+	−
CP-5	+	+	+	SP-5	+	+	−
CP-6	+	+	+	SP-6	+	+	+
CP-7	+	+	−	SP-7	+	+	+
CP-8	+	+	−	SP-8	+	+	+
CP-9	+	+	−	SP-9	+	+	−
CP-10	+	+	+	SP-10	+	+	−
CP-11	+	+	−	SP-11	+	+	+
CP-12	+	+	+	SP-12	+	+	−
CP-13	+	+	+	SP-13	+	+	−
CP-14	+	+	−	SP-14	+	+	+
CP-15	+	+	−	SP-15	+	+	−
Lindao 10	−	−	−	SP-16	+	+	−

described in the DIG Applications Manual for Filter Hybridisation (Roche Diagnostics GmbH, Mannheim, Germany) to detect the integration of the target gene into the rice genome.

2.5 *Total RNA extraction and Northern blot analysis*

Total RNA was extracted from selected transgenic rice lines and from a wild-type rice plant using TRIzol Reagent (Invitrogen). A total of 20 μg of total RNA for each sample was fractionated via electrophoresis on a 1.2% denaturing formaldehyde gel using 1×MOPS buffer, and the RNA was transferred onto a Hybond-N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK). Northern hybridization was carried out using transgene-specific digoxigenin-labelled RNA probes. The RNA probes corresponding to RSV *CP* and RSV *SP* were prepared with T_7 RNA polymerase using a DIG RNA Labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). Northern blotting was performed according to the manufacturer's instructions.

2.6 *Small interfering RNAs (siRNA) extraction and Northern blot analysis*

siRNAs were extracted from the transgenic rice lines and a wild-type plant using a PureLink™ miRNA Isolation Kit (Invitrogen), according to the manufacturer's instructions. Approximately 5 μg of small RNA was fractionated on a 15% polyacrylamide gel with 7 M urea, and the small RNAs was transferred onto a Hybond-N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK) following the same procedure and using the same probes used for total RNA analysis.

2.7 *Assessment of resistance to RSV*

Viral resistance assays were carried out using viruliferous insects (*Laodelphgax striatellus*). T_2 transgenic rice seedlings in the five-leaf to ten-leaf stage were exposed to approximately four nymphs per plant in an inoculation cage. The insects were brushed off the plants twice a day to achieve uniform infection rates. Insects were killed using insecticide 72 h post inoculation (p.i.), and the plants were transferred to an insect-free field under natural sunlight for further assessment. The appearance of symptoms on the developing leaves was assessed after 1 week up to 7 weeks.

3. Results and analysis

3.1 *IR constructs and transformation of plants*

The standard transformation plasmid pCAMBIA 1300 was modified by inserting the NOS terminator left border–right border segment to obtain the twin T-DNA vector, one of which encoded the *hpt* gene. IR constructs targeting the 400-nucleotide region of either the CP or the SP genome segments of RSV were assembled into the other T-DNA. A Ubi promoter and a PLD intron were used in the IR constructs to produce high levels of the dsRNA in transgenic rice (Smith *et al.*

Table 3. PCR analysis of T_1 transformation events derived from pDTRSVCP and pDTRSVSP

Transformed vector	Plant no. (T_0)	Number of T_1 plants			
		T+/H+	T+/H−	T−/H+	T−/H−
pDTRSVCP	CP-1	10	0	5	3
	CP-3	9	4	4	8
	CP-5	11	0	0	6
	CP-6	9	2	3	5
	CP-10	12	3	4	3
	CP-12	15	4	6	4
	CP-13	14	0	0	5
pDTRSVSP	SP-2	12	0	0	5
	SP-3	11	3	6	3
	SP-6	10	4	3	2
	SP-7	14	2	0	2
	SP-8	13	2	2	0
	SP-11	17	4	8	7
	SP-14	9	3	4	0

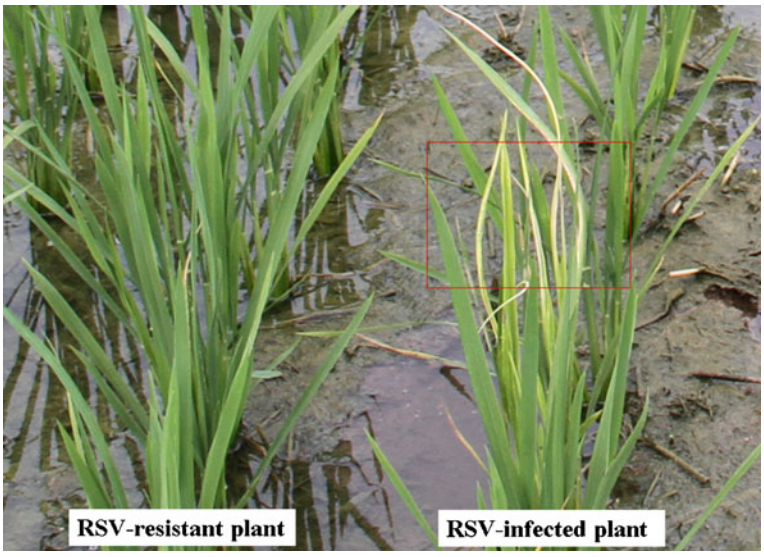

Figure 2. Symptoms of RSV in the transgenic resistant plants and the infected control plant. The RSV-resistant plant (left) showed no symptoms of RSV infection in the resistant transgenic plant at 30 days p.i., whereas the RSV-infected plant (right) showed typical symptoms in the control plant at 30 days p.i. The symptoms of RSV infection include chlorotic stripes, mottled leaves, and withered inner leaves as shown in the red box within the picture.

2000; Tyagi and Mohanty 2000). We introduced each IR construct of the twin T-DNA vector into the rice calli (cv. Lindao 10).

3.2 *Selection and analysis of T_0 transgenic plants*

All hygromycin-resistant (H-R) calli from cv Lindao 10 were placed on regeneration medium containing hygromycin B (50 mg/L) to obtain T_0 plants. The selected transformants were analysed for hygromycin tolerance using leaf painting assays, according to a previously published method (Breitler *et al.* 2004). PCR assays confirmed that all H-R transformants were *hpt*-positive. Among the 15 primary transformants derived from pDTRSVCP that were selected using hygromycin B, 7 individuals were CP-positive. Of the 16 primary transformants derived from pDTRSVSP, 7 were SP-positive (table 2).

Table 4. Results of RSV resistance to RSV of T2 transgenic rice lines including pDTRSVCP and pDTRSVSP

Transgenic Lines	Number of inoculated plants	Number of RSV susceptive plants	Percentage of susceptible plants (%)
CP-3-T2-2	50	2	4
CP-6-T2-1	50	9	18
CP-10-T2-3	50	2	4
CP-12-T2-2	50	12	24
SP-3-T2-2	50	1	2
SP-6-T2-3	50	11	22
SP-7-T2-1	50	0	0
SP-8-T2-2	50	13	26
SP-11-T2-1	50	8	16
SP-14-T2-3	50	2	4
cv. Lindao 10	100	38	38

3.3 *Segregation of the marker gene in the T_1 progeny*

Primary transformants that harboured both the *hpt* gene and the target gene (CP or SP of RSV) were selfed to obtained T_1 transgenic plants. All T_1 plants were simultaneously analysed via PCR and leaf painting assays. PCR assays verified that target gene-positive and *hpt*-negative progeny were obtained from 7 of the 15 plants transformed with pDTRSVCP and from 7 out of the 16 plants transformed with pDTRSVSP. We obtained 4 and 6 lines that were target gene-positive and *hpt*-negative (T+/H−) in the pDTRSVCP and pDTRSVSP T_1 progeny, respectively. Of the entire detected pDTRSVCP T_1 population, 13 plants were T+/H−, and in the whole detected pDTRSVSP T_1 population, 18 plants were T+/H− (table 3).

3.4 *T_2 transgenic rice plants displayed increased high-level resistance to RSV*

Selected T_1 plants (T+/H−) were selfed to obtain the T_2 progeny. All T_2 rice plants in the five-leaf to ten-leaf stage were analysed for hygromycin-tolerance using leaf painting assays. Up to 50 plants from each T_2 line were inoculated with the RSV isolates using viruliferous vector insects, with wild-type

rice (cv. Lindao 10) serving as the control. The plants were monitored daily for the appearance of symptoms. At 30 days p.i., the susceptible rice plant exhibited typical symptoms, including chlorotic stripes, mottled leaves, and withered inner leaves; however, the resistant transgenic plants did not exhibit any symptoms of infection (figure 2). The CP-3-T2-2 and CP-10-T2-3 lines of pDTRSVCP and SP-3-T2-2, SP-7-T2-1, and SP-14-T2-3 lines of pDTRSVSP transgenic plants exhibited significant resistance to RSV, with a susceptibility ratio of less than 5%, which was far lower than that of wild-type rice (38%). The resistance of other transgenic ranged from moderate to no resistance, with susceptible ratios greater than 16% (Zhou *et al.* 2011; table 4).

3.5 *Molecular analysis of resistant transgenic T_2 progeny*

Genomic DNA extracted from the RSV-resistant T_2 plants was digested with *Hin*d III and sequentially hybridized to the target gene. Figure 3 shows Southern blot analysis of T_2 resistant transgenic plants. The RSV CP target hybridizing signal was observed in the lines CP-3-T2-2 and CP-10-T2-3 lines, and the RSV SP target hybridizing signal was observed in the SP-3-T2-2, SP-7-T2-1 and SP-14-T2-3 lines. CP-3-T2-2 and SP-7-T2-1 harboured a single-copy of the target genes, and lines CP-10-T2-3, SP-3-T2-2 and SP-14-T2-3 harboured two copies of the target genes, respectively. Southern blotting revealed that the target genes (RSV CP or SP) were stably integrated into the rice genome in the resistant transgenic plants at low copy numbers.

To monitor the extent of RNA silencing in the transgenic plants, total RNA and siRNA were extracted from the T_2 plants and sequentially analysed via Northern blotting using transgene-specific RNA probes. Compared with wild-type rice, the target genes were transcribed in the T_2 transgenic progeny. The expected siRNAs were observed in the transgenic rice plants, but not in the wild-type rice plant (figure 4).

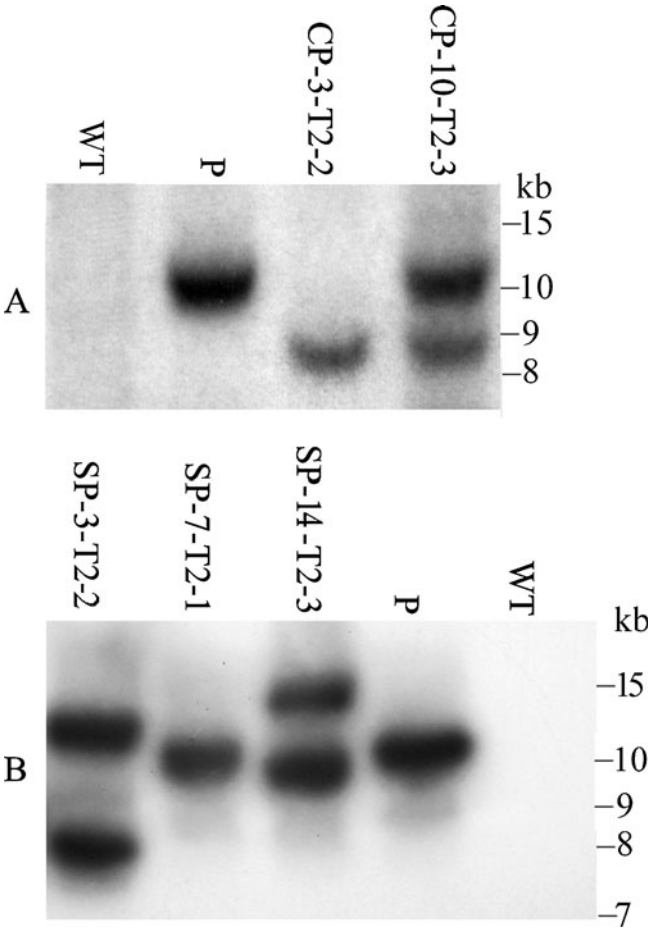

Figure 3. Southern blot analysis of T_2 transgenic plants resistant to RSV. (**A**) Southern blot analysis of total DNA from T_2 pDTRSVCP transgenic plants. (**B**) Southern blot analysis of total DNA from T_2 pDTRSVSP transgenic plants. DNA from a non-transgenic plant (WT) and the transformants were digested with *Hind*III, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the RSV CP and SP probes. Lanes CP-3-T2-2, CP-10-T2-3, lanes SP-3-T2-2, SP-7-T2-1, SP-14-T2-3: resistant T_2 plants of transgenic lines. WT: wild-type, non-transgenic plant; P: pDTRSVCP plasmid (**A**) and pDTRSVSP plasmid (**B**).

4. Discussion

Several genetic-engineering-based strategies have been applied to control RSV. Hayakawa *et al.* (1992) and Yan *et al.* (1997) introduced the RSV CP gene into rice, and their results suggested that CP-mediated resistance to RSV infection could be introduced in transgenic plants. Protein-mediated resistance often confers moderate resistance, whereas RMVR often confers high levels of resistance or even complete viral immunity (Prins 2003). Not all RNAi constructs against viral RNAs are equally effective for RMVR. Targeting the gene that encodes a protein that is critical for viral proliferation is a practical and effective way to control viral infection in crop plants (Shimizu *et al.* 2009; Shimizu *et al.* 2011). Shimizu *et al.* (2011) analysed RNAi-mediated resistance to RSV using RNAi targeting each coding gene in the RSV genome. They observed high-level RSV-resistance in transgenic plants expressing the CP gene (pC3), and a lack of RSV-resistance in those that expressed the SP gene (pC4). However, recent studies indicated targeting both the CP and SP genes increases RSV resistance (Ma *et al.* 2011; Zhou. *et al.* 2012), likely due to different linkers and origins of the stem sequences in the IR structures. Previous studies revealed that constructs encoding intron-hpRNA induced PTGS at high efficiency due to the intron excision process from the construct by the spliceosome (Smith *et al.* 2000). Meanwhile, the stem sequences of the hairpin from different origins may influence the viral resistance level

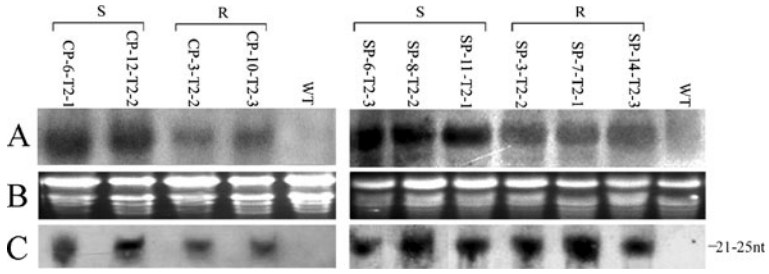

Figure 4. Northern blot analysis of total RNA and siRNA from plants of each T_2 generation transgenic lines. (**A**) Result of northern blot analysis of total RNA. (**B**) Normalized RNA in each lane. (**C**) Result of Northern blot analysis of siRNA. Total RNA (10 μg) and low-molecular-weight RNA (5 μg) extracted from each of the representative plants shown in table 4. The total RNA and siRNA was hybridized with DIG-labelled RSV CP or SP RNA probes, respectively. Lines: S: Susceptible lines; R: Resistant lines; WT: Wild type plant.

of hairpin-expressing plants (Luo and Chang 2004; Jiang *et al.* 2011). Here, we show that IR constructs containing either the CP or the SP genes of RSV flanking the PLD intron efficiently induces resistance to RSV inoculation.

Considering the possible large-scale commercial planting of such transgenic crops, transgenic crops that do not contain selection markers are desired. The integration of the selected marker gene (*hpt*) and the gene of interest (RSV CP or SP) is crucial so that these two genes can be separated in the T_1 generation. In primary transformants of pDTRSVCP and pDTRSVSP, integration of the *hpt* marker gene and the target gene (RSV CP or SP) occurred in 7 events each. Subsequently, in the entire detected T_1 population, we obtained 13 T+/H− plants of pDTRSVCP and 18 T+/H− plants of pDTRSVSP (table 3). If the two T-DNAs behaved as independent Mendelian loci, the expected segregation ratio in the T_1 generation would be 9:3:3:1 for T+/H+, T+/H−, T−/H+ and T−/H− events (Xing *et al.* 2000). However, in our study, the ratios could not be calculated because the data obtained for each events in the T_1 plants were insufficient for a significant statistical analysis. The inheritance of foreign alleles should be assessed in subsequent generations that are stable and homozygous.

Transgene expression instability is a common phenomenon in modified plants from monocot species (Iyer *et al.* 2000). Transgene stability is affected by multiple factors, including the structure and position of the transgene, interaction of the transgene with the endogenous genes, and its copy number. A single intact copy that integrates into the transgenic plant genome is usually desirable, as it reduces the potential for unintended insertional inactivation events (Koprek *et al.* 2001; Meng *et al.* 2006; Jackson *et al.* 2013). Transgenic plants with low copy numbers displayed a recovery phenotype in RMVR, and high resistance could be achieved with no viral infection or symptoms in the new leaf tissue (Goodwin *et al.* 1996; Zhu *et al.* 2005). In the current study, the marker-free T_1 plants were selfed to obtain a T_2 progeny, which displayed stable integration of the target gene at low copy numbers (figure 3).

We performed a Northern blot analysis to determine if the resistance level of the transgenic plants correlated with the siRNA expression level. The results revealed that siRNA was present in all the transgenic plants, whereas no siRNA was observed in the wild-type plants (figure 4). As previously reported, the accumulation of sequence-specific siRNAs did not correlate with the level of viral resistance (Qu *et al.* 2007; Jiang *et al.* 2011). Not all siRNA created from transgenes are equally effective for RMVR, despite similar total siRNA content.

The growth and development of the transgenic plants were not significantly different from wild-type rice. Our results may help increase the rice harvest in areas where RSV is a problem, and we provide a strategy to generate selectable marker-free transgenic rice with strong heritable RSV resistance that are applicable to large-scale commercial planting.

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