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Induction of RNA-mediated Resistance to Papaya Ringspot Virus Type W

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Transformation of cantaloupes with the coat protein (*cp*) gene of papaya ringspot virus type W (PRSV-W), Thai isolate, was used to introduce virus resistance. Binary vectors containing either the full length coat protein coding region under control of the *35S CaMV* promoter (pSA1175), or the inverted-repeat of a coat protein coding region (pSA1304), were constructed and used for *Agrobacterium*-mediated transformation of cotyledonary explants of the cantaloupe cultivar Sun Lady. Four independent transgenic lines were obtained using pSA1304 and one using pSA1175. Integration of the PRSV-W *cp* gene into the genome of these transgenic lines was verified by PCR amplification, GUS assays and Southern blot hybridization. *In vitro* inoculation of these lines with PRSV-W revealed that whereas the line containing pSA1175 remained sensitive, the four lines containing pSA1304 were resistant. The presence of small RNA species, presumably siRNA, corresponding to regions of the viral *cp* gene in transgenic lines resistant to PRSV-W supports the involvement of post-transcriptional gene silencing in the establishment of resistance.

Keywords: Inverted-repeat, *In vitro* inoculation, PRSV type W, Pathogen-derived resistance (PDR), siRNA, Virus resistance

Introduction

Cucurbit species include a variety of high value crops e.g., melons, watermelon, cucumber, cantaloupe, summer squashes, and winter squashes that grow in tropical and subtropical areas around the world. Major losses of cucurbit production result from infection by several viruses including zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), papaya ringspot virus type W (PRSV type W) and cucumber mosaic virus (CMV). Control of these viruses by insecticides is ineffective as there are several insect vectors, such as aphids which can transmit the virus within a very short period. The conventional incorporation of viral resistance genes derived from wild species is not easy because these genes are not simply inherited or are recessive (Bernadac *et al*., 2002). The concept of pathogen-derived resistance (PDR) was proposed in 1985 (Sanford and Johnston, 1985) and genetic engineering technologies have enabled the introduction of virus-derived genes such as coat protein, replicase and helper component protein into plants to confer virus resistance. In the present study, we have investigated the use of the PRSV type W *cp* gene to confer virus resistance to cantaloupe.

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Materials and methods

Plasmid construction. Standard DNA cloning methods (Sambrook *et al*., 1989) were used for plasmid construction. Amplification of cDNA corresponding to the full-length coat protein coding region (positions 9269 to 10210 of PRSV-W genome sequence, accession number AY10722, GenBank) was from the RNA of PRSV type W isolate Ratchaburi by RT-PCR using PRV-5/PRV-3 primers (Table 1, Fig. 1C). These primers introduced the *Nco*I sites at the 5' and 3' ends of the amplified *cp* gene which were used for the insertion of the amplified *cp* gene between the 35SCaMV promoter and 35SCaMV polyA signal of plasmid pPRT103 (Tepfer *et al*., 1987). The translational enhancer of alfalfa mosaic virus (AMV) (Gallie *et al*., 1987) was inserted into the *Kpn*I site between the 35SCaMV promoter region and the *cp* gene. The *Hin*dIII fragment containing the CP expression cassette was isolated and inserted at the *Hin*dIII site between the *nptII* and the *gus* genes of T-DNA to generate plasmid pSA1175 (Fig. 1A).

The binary vector pSA1304 has the same vector backbone as pSA1175 except for the *cp* gene cassette which was replaced with the inverted repeat of the 669 bp of the *cp* gene (position 9464 to 10132 of PRSV type W genome sequence) and amplified using CPN-M/CPB-3 primers (Table 1, Fig. 1C). Intron number 12 of the NADH-dependent hydroxypyruvate reductase (*hpr-A*) (accession number X58542, GenBank) was inserted between the sense and antisense fragments of

Table 1. Oligonucleotide primers used for PCR amplification. The *Nco*I/*Bam*HI restriction sites are underlined

Primer	Primer Sequences
PRV-5	5'-ATCATTCCATGGCTGTGGATGCTGGTTTGAATG-3' (*Nco*I)
PRV-3	5'-GTCAAGCCATGGTTGCGCAGCCACACTGTATTCTTATG-3' (*Nco*I)
CPN-M	5'-TTTACTGATACCATGGTTTTACCAAG-3' (*Nco*I)
CPB-3	5'-CACTAGCGCCAGGATCCAATTG-3' (*Bam*HI)
INT-F	5'-AACAGGATCCTCCCTAACTGAAAAG-3' (*Bam*HI)
INT-R	5'-TCACAGCTTTGAAGTAGAGGATCCTG-3' (*Bam*HI)
CPM-F	5'-ACATCTCAAACACTCGTGCCA-3'
CPM2-R	5'-CTTTGTTGACATCTTCCACTG-3'
NPT-F	5'-ACAAGATGGATTGCACGCAGGTTC-3'
NPT-R	5'-GAACTCGTCAAGAAGGCGATAGAAGG-3'
REP-F	5'-GACTGTGGGCTATGGAGGATTGG-3'
REP-R	5'-CGAAGGATCCTAGTTGACACATCGATTC-3'
ACT-F	5'-AACGGGAAATTGTCCGTGAC-3'
ACT-R	5'-ATCTGCTGGAAGGTGCTTAG-3'

the partial *cp* gene (Fig. 1B). This intron was amplified from cucumber genomic DNA using the INT-F/INT-R primers (Table 1).

These binary vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404 by a freeze-thaw method (Hofgen and Willmitzer, 1988).

Plant transformation. Cantaloupe (*Cucumis melo* L. var. *Cantalupensis* cv. Sun Lady) was germinated on MS medium (Murashige and Skoog, 1962). Cotyledons from four day old axenic cantaloupe seedlings were transformed with *Agrobacterium tumefaciens* as described by Gonsalves *et al*. (1994). The proximal and distal ends were removed and the cotyledon was transversely cut into two parts. The explants were incubated in an overnight culture of *Agrobacterium* for 20 min. The inoculated explants were blotted dry and placed abaxial side up onto the shoot induction medium containing MS medium supplemented with 1 mg/l benzylaminopurine (BAP) and 2 mg/l 3',5'-dimethoxy-4'-hydroxyacetophenone (a derivative of acetosyringone, Aldrich). The explants were co-cultivated at 28°C in the dark for 3 days and transferred to the 8 hr dark/ 16 hr light cycle on the selective shoot induction medium containing MS medium supplemented with 1 mg/l benzylaminopurine (BAP), 100 mg/l kanamycin and 500 mg/l claforan. After 2 to 3 weeks, the regenerated shoots were excised and subcultured on the selective elongation medium containing MS medium supplemented with 0.05 mg/l benzylaminopurine (BAP), 100 mg/l kanamycin and 500 mg/l claforan for another 2 to 3 weeks. The elongated shoots were excised and transferred to rooting medium containing MS medium supplemented with 0.01 mg/l naphthalene acetic acid (NAA) and 250 mg/l claforan. The plantlets were *in vitro* propagated and acclimatized in a greenhouse.

Polymerase chain reaction (PCR). Total genomic DNA was extracted from young plant leaves by CTAB method (Guillemaut and Marechal-Drouard, 1992). The 50 µl of total PCR reaction was composed of 100 ng DNA template, 50 pmol of each forward and reverse primers, 200 µM of dNTPs mix, 1× PCR buffer (20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 100 µg/ml nuclease-free BSA), 0.5 µl (1.5 units) *Taq* DNA polymerase and water. The amplification was carried out in the Perkin-Elmer Thermal Cycler 2400 under the following condition: 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, for 30 cycles. PCR products were analyzed by agarose gel electrophoresis. The DNA fragment of *cp* gene was amplified using the CPM-F/CPM2-R primers (Table 1, Fig. 1C) and the DNA fragment of neomycin phosphoryl transferase II (*nptII*) gene was amplified using the NPT-F/ NPT-R primers (Table 1).

Southern blot hybridization. Ten micrograms of plant total DNA were digested with *Hin*dIII. The digested DNA fragments were separated using 0.8% agarose gel electrophoresis and transferred to Hybond-N+ membrane (Amersham). The membrane was hybridized with the 669 bp fragment of *cp* gene labeled with PCR DIG probe synthesis kit (Roche) and detected with CDP-Star* according to the manufacturer's instruction. After hybridization, the membrane was washed and re-hybridized with the labeled DNA probe specific to *gus* gene.

Northern blot hybridization. Total RNA was extracted from plant leaf as described by Hamilton and Baulcombe (1999) and Szittya *et al.* (2002). The lower molecular weight RNAs fractions were recovered from the supernatant and separated on 15% polyacrylamide gel and transferred to Hybond N+ membrane (Amersham) by electroblotting with Trans-Blot® SD Semi-Dry Transfercell (Bio-Rad). Northern blot hybridization was performed as in Hamilton and Baulcombe (1999) and Szittya *et al.* (2002). The RNA probe was the 669 bp fragment of *cp* gene. This fragment was amplified by PCR and

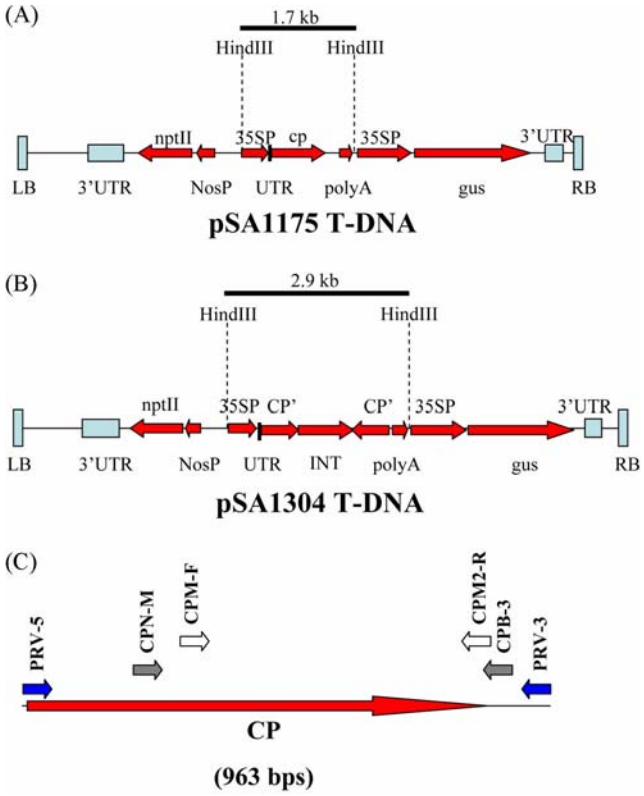

Fig. 1. Organization of the binary vector T-DNA regions. (A) pSA1175 contains the full length PRSV-W coat protein (CP) coding region under the control of the CaMV35S promoter (35SP) and the 35S CaMV terminator (35ST) and NPTII and GUS expression cassettes. (B) pSA1304 contains two copies of 668 bp of the CP coding region in sense and antisense orientations separated by a cucumber gene intron (INT). (**C**) Primers used to amplify specific coding regions of the PRSV-W coat protein. LB: left border, RB: right border, 3' UTR: 3' untranslated region; *nptII*: neomycin phosphotransferase II gene; Nos P: nopaline synthase promoter; 35S P: CaMV 35S promoter; *cp*: full length PRSV-W coat protein; *cp*': partial PRSV-W coat protein gene; polyA: polyadenylation signal; *gus*: β-glucuronidase gene;

inserted into the pGEM-3zf(+) plasmid (Promega) after the T7 promoter. The plasmid was linearized with *Sph*I and purified. The RNA probe was synthesized by *in vitro* transcription according to Promega protocol and labeled with [α^{32}P]UTP. The DNA was eliminated from the probe using RNase-free DNase. The RNA probe was hydrolyzed in carbonate buffer to obtain the average size of about 50 nucleotides, neutralized with 3 M sodium acetate, pH 5.0 and used for hybridization with the lower molecular weight RNAs in the prepared filter.

GUS Histochemical assay. The β-d-glucuronidase (GUS) expression in plant tissue was detected using 5-bromo-4-chloro-3-indoyl-β-d-glucuronic acid as substrate (Jefferson *et al*., 1987). Leaf tissues of 0.5 cm^2 size were immersed in substrate solution in an eppendorf tube and incubated overnight at 37°C. Chlorophyll was removed with 95% ethanol.

Mechanical inoculation of in *vitro* plantlets. PRSV type W isolate Ratchaburi was propagated in wild type pumpkin plants (*Cucumis pepo*). Sap extracts from virus-infected pumpkin leaf were diluted 1 : 5 in 0.1 M phosphate buffer, pH 7.0, and centrifuged at 10,000 × g for 2 min. The supernatant was passed through a 0.2 µ filter to remove bacterial and fungal contaminants. Mechanical inoculations were done twice aseptically in a laminar flow hood as described by Russo and Slack (1998) and Mazier *et al*. (2004). The sterilized celite was applied on the first leaf from the top of plantlet together with 10 µl of sap extract. The sterilized cotton swap was rubbed over the surface of leaf using sterilized spatula to support under of leaf. Mock inoculation was performed using phosphate buffer on a non-transformed plantlet. The second inoculation was performed 5 days after the first inoculation on the newly emerged leaf next to the first inoculated leaf.

Inoculated plants were returned to *in vitro* growth conditions for 14 days. The new growth leaf was tested for the presence of virus by RT-PCR using the PRSV-W replicase (*NIb*) gene specific primers REP-F/REP-R (Table 1). The actin gene specific primers ACT-F/ACT-R (Table 1) were used as an internal control of mRNA in the same RT-PCR reaction. Infected plants were considered from detection of PRSV-W by RT-PCR. The data from virus infected plants was statistical analyzed using SPSS.

Results

Molecular analysis of transgenic plants. Transformation of cantaloupe cotyledon explants with *Agrobacterium* containing pSA1175 and pSA1304 yielded 8 and 12 putatively transgenic plantlets, respectively. As selection on kanamycin (25 mg/l for 2-3 weeks) was detrimental to rooting, the putative transgenic plantlets were *in vitro*-propagated (sub-cloned) in non-selective media for analysis.

Two primer sets specific to the *nptII* and *cp* genes were used for verification that the regenerated plants were transgenic. A fragment of 781 bp is predicted to be amplified using the *nptII* specific primers whereas the *cp* gene-specific primers should amplify a fragment of 534 bp. The results (Fig. 2) revealed that one transgenic line (S1) was obtained from the explants transformed with *Agrobacterium* harboring plasmid pSA1175 and four transgenic lines (IR1, IR2, IR3 and IR4) were obtained from the explants transformed with *Agrobacterium* harboring plasmid pSA1304.

Histochemical staining of leaves from the regenerated plantlets confirmed that all five transgenic lines contained a *gus* gene insert (Fig. 3). Southern blot analysis of the total plant DNA digested with *Hin*dIII hybridized with DNA probes specific to the *cp* gene (Fig. 4A) and the *gus* gene (Fig. 4b) revealed that all 5 transgenic lines contain *cp* and *gus* genes (Fig. 4A and B). Hybridization using the *cp* gene specific probe showed a single 1.7 kb band in transgenic line S1 and a 2.9 kb band in transgenic lines IR1, IR2, IR3 and IR4. These two bands correspond to the *cp* expression cassette in the T-DNA of pSA1175 and pSA1304, respectively. Additional hybridizing bands can be seen in lines IR2, IR3 and IR4, indicating that some rearrangement had occurred.

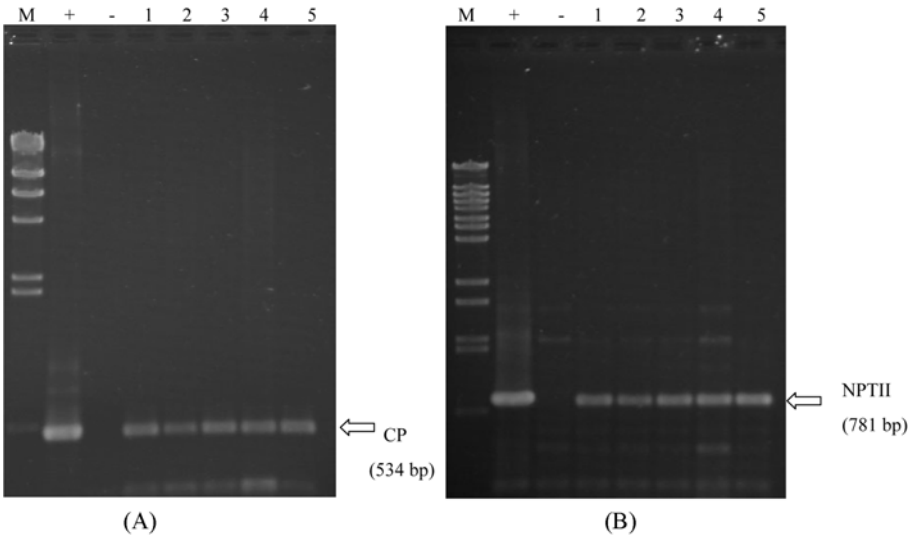

Fig. 2. Verification of cantaloupe transformation. The presence of the partial CP coding region (534 bp) and (B) the NPTII coding region (781 bp) was confirmed by PCR amplification from genomic DNA isolated from transgenic plant lines S1 (pSA1175), IR1 (pSA1304), IR2 (pSA1304), IR3 (pSA1304) and IR4 (pSA1304). DNA from plasmid pSA1304 and a non-transformed plant were used as positive (+) and negative (-) controls, respectively. M1: λ-DNA digested with *Hin*dIII, M2: λ-DNA digested with *Bst*EII

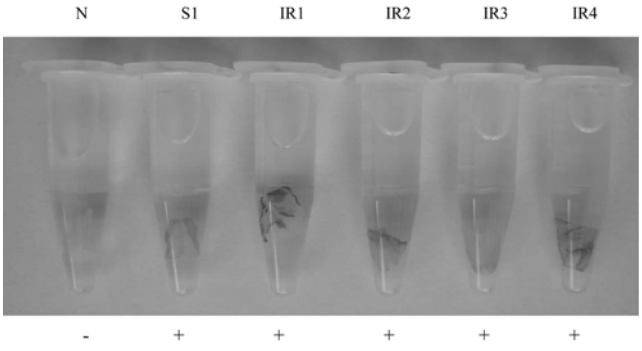

Fig. 3. GUS histochemical staining of transgenic cantaloupe leaves. The blue color observed for cantaloupe lines S1, IR1, IR2, IR3 and IR4 confirmed the presence of the GUS expression cassette. N is a non-transformed cantaloupe leaf for which no blue color was observed.

The profile also suggested that lines IR2 and IR4 could be siblings.

Resistance of transgenic plantlets to inoculation by PRSV-W. The ability of the transgenic cantaloupe plantlets to resist infection by PRSV type W infection was tested in 9 plantlets of line S1, 3 plantlets of line IR1 and 8 plantlets of each of the lines IR2, IR3 and IR4. Fourteen days after *in vitro* inoculation, the infection was examined in the leaf above the inoculated leaf by RT-PCR, using primers specific to the PRSV type W replicase gene. Leaves of all non-transformed plantlets were found to be infected. Five of the 9 plants of the transgenic line S1 were infected but no systemic virus infection was detected in inoculated plants of transgenic lines IR1, IR2, IR3 and IR4 (Table 2, Fig. 5A, 5B and 5C). From chi-square analysis, transgenic line S1 was considered to be susceptible to PRSV-W ($p > 0.05$) while transgenic lines IR1-IR4 were considered to be resistance to PRSV-W infection ($p < 0.05$).

Detection of coat protein specific siRNAs. The presence of siRNAs has been reported as a hallmark of posttranscriptional gene silencing (Hamilton and Baulcombe, 1999). To investigate the correlation between the presence of siRNAs and the PRSV-W resistance of transgenic plants, low molecular weightl RNAs were isolated from all transgenic lines and probed with RNA probe specific to the *cp* gene. The band comprises of 21-23 bp small RNAs specific to the regions of the PRSV-W was obviously detected in all 4 transgenic lines resistance to PRSV-W (Fig. 6). A non specific weak band of smaller size was present in transgenic line S1.

Discussion

In this study, we report the development of transgenic cantaloupe plants resistant to papaya ringspot virus type W (PRSV-W). The first plasmid construct, pSA1175, containing the full length of the *cp* gene of PRSV-W is aimed for *cp* gene expression in transgenic cantaloupe while the second plasmid construct, pSA1304, that expresses an inverted-repeat of part of the CP coding region, is designed for induction of post transcriptional gene silencing by RNA interference (RNAi) in transgenic cantaloupe. The frequency of *Agrobacterium*-mediated transformation into the cotyledons of cantaloupe cv. Sun Lady is about 1%, consistent with previously reported melon transformation frequencies of 1% (Gaba *et al*., 1992, Gonsalves *et al.*, 1994) to 7% (Fang and Grumet, 1990). As

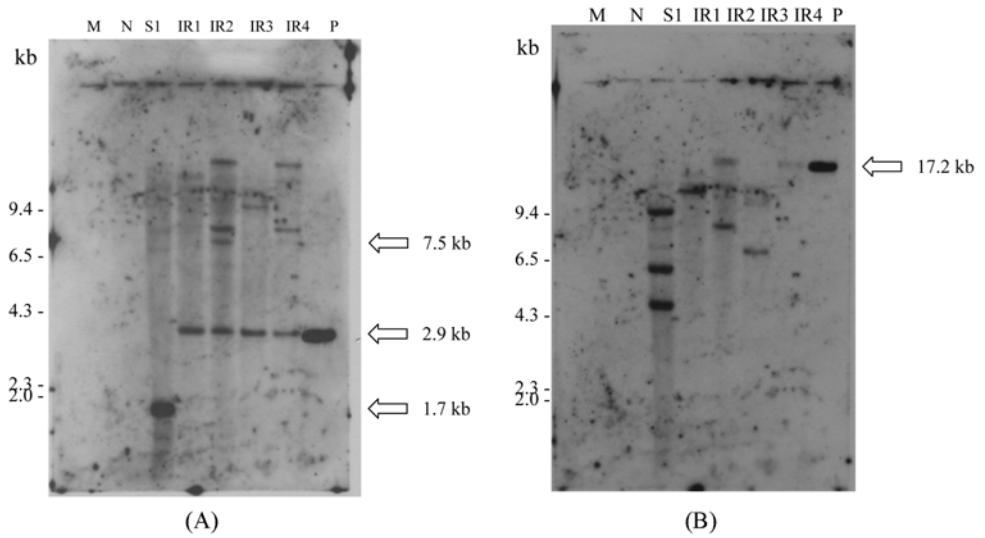

Fig. 4. Southern blot analysis of transgenic cantaloupe. Genomic DNA from transformed lines S1, IR1, IR2, IR3, IR4 and non-transformed line N was digested with *Hin*dIII, separated on agarose gel, transferred to nylon membrane and probed with DNA amplified from (A) the CP coding region and, after washing and drying, (B) the GUS coding region. Lane M was loaded λ-DNA digested with *Hin*dIII as a size marker and Lane P contained *Hin*dIII-digested pSA1304.

Table 2. Susceptibility of transgenic cantaloupe to infection by PRSV-W

Line	Total plant	No. infected	% infected
Untransformed plants	6	6	100
S1	9	5	63.6
IR1	3	0	0
IR2	8	0	0
IR3	8	0	0
IR4	8	0	0

cucurbit plants are reported to be recalcitrant for regeneration in tissue culture (Fassuliotis and Nelson, 1992), the ability to transform cucurbits is rather variable (Gaba *et al*., 1996). Even though the transformation is performed using the same cultivar from the same source and by the same procedures, it is not easily repeatable in different laboratories (Gaba *et al*., 2004). We used *npt*II gene as a selectable marker and kanamycin for transgenic plant selection in our transformation. Only 12.5% (1 from 8) and 33.3% (4 from 12) of regenerated plants were verified as transgenic. The high number of escape plants probably resulted from the *npt*II selectable marker as it was reported that melon cotyledon is moderately resistant to kanamycin (Dong *et al*., 1991). In the future, other selectable markers, such as the dihydrofolate reductase gene (Dong *et al*., 1991) can be used to improve the efficiency of transgenic plant selection.

Molecular analysis of transgenic plants indicated that rearrangement of the transgene had occurred in some of them. This rearrangement, however, did not affect the expression of GUS activity. While all four transgenic lines containing pSA1304 exhibited high levels of resistance to PRSV-W infection, the transgenic line containing pSA1175 was susceptible to infection by PRSV-W. No traces of virus could be detected in PRSV-W resistant plants by molecular methods. In the process of post-transcriptional gene silencing (PTGS), the inverted-repeat construct of the target gene encodes a self complementary hairpin RNA (hpRNA) or double stranded RNA (dsRNA). The dsRNA is cleaved by DICER, a member of the RNase III family (Bernstein *et al*., 2001), into several 21-25 nucleotide double-stranded RNAs termed small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Zamore *et al*., 2000; Elbashir *et al*., 2001). After unwinding, these single stranded RNAs incorporate into the RNA-induced silencing complexes (RISCs), trigger the surveillance mechanism. This induces a specific RNA degradation process leading to the destruction of the transgene RNA that has a cognate sequence to that of the siRNA and subsequently to the entire viral genome (Smith *et al*., 2000; Wesley *et al*., 2001; Vaucheret *et al*., 2001, Chen *et al*., 2004). Northern blot detection of 21-23 nt RNAs specific to the PRSV-W CP coding region in all four transgenic lines resistant to PRSV-W indicated that the viral-derived CP hairpin construct had a high efficiency (100%) for inducing RNA-mediated silencing to protect the cantaloupe against PRSV-W infection. It might be possible to use the 23 bp siRNA to confer resistance to PRSV-W in transgenic plant, however this might not be effective as size constraints and sequence specificity of the plant virus sequences to induce PTGS in the host plant are known to exist (Pang *et al*, 1987, Jan *et al*, 2000, Waterhouse and Helliwell, 2003).

The expression of the *cp* gene from different viruses in transgenic plants exhibits different roles. In several cases, *cp* gene expression protects the transgenic plants from viral

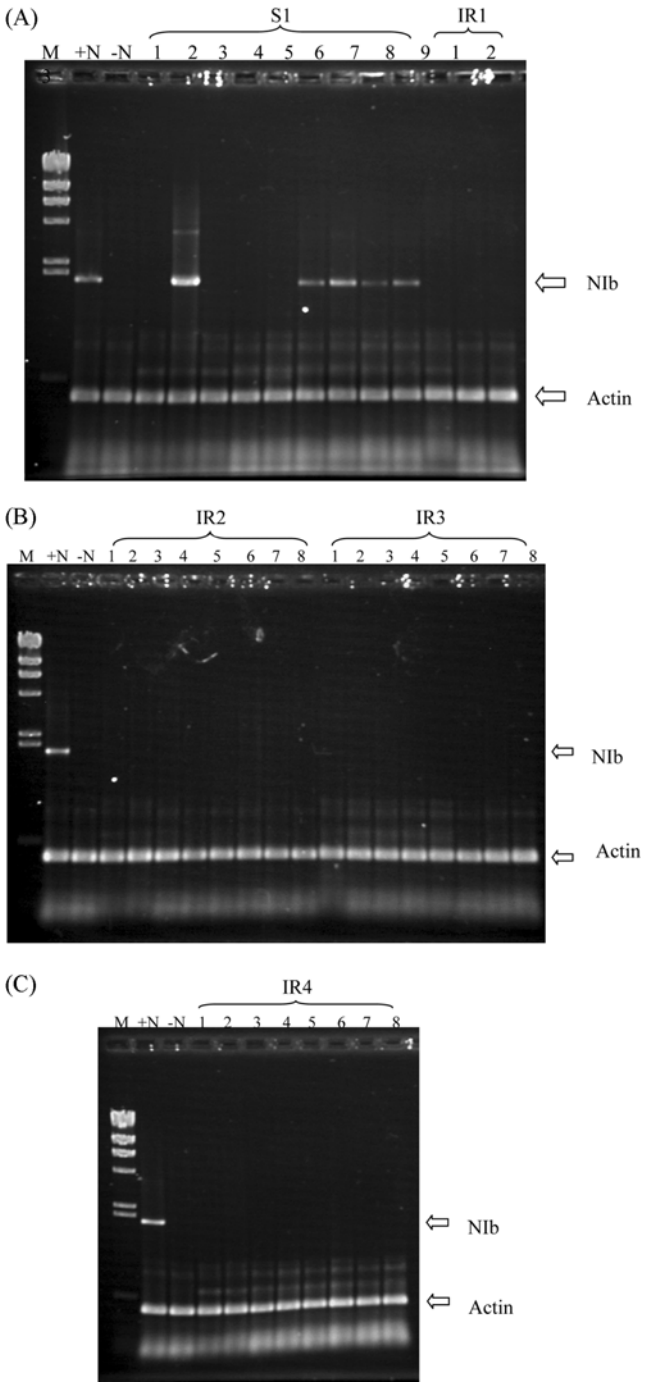

Fig. 5. Evidence for resistance to PRSV-W in transgenic cantaloupe. RT-PCR-amplification of DNA from the PRSV-W replicase gene (*NIb*) following viral inoculation of (panel A) SI transformant (subclones 1-9) and IR1 transformant (subclones 1-3) was used to show resistance (no signal) in S1 subclones 1,3,4,5 and in IR1 subclones 1-3. Panel B shows that all subclones of plants IR2 and 3 are resistant to PRSV-W. Panel **C** shows that all subclones of plant IR4 are resistant to PRSV-W. RT-PCR amplification of actin mRNA was used to show an equal loading of RNA template for each sample. M: λ-DNA digested with *Hin*dIII; +N: inoculated non-transgenic line (signal shows infection), -N: mock inoculated non-transgenic line.

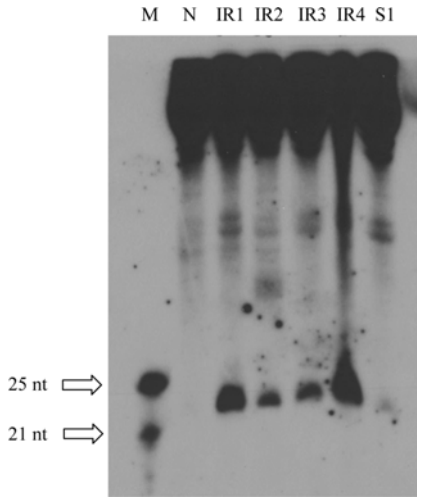

Fig. 6. Evidence that resistance to PRSV-W involves the RNAi pathway. Low molecular weight RNAs were obtained from a non-transgenic line (N) and from transgenic lines IR1, IR2, IR3, IR4 and S1 were separated on a 15% polyacrylamide gel. After transfer to Hybond N+ membrane, the presence of siRNAs was detected by hybridization with single-stranded P^{32}-labeled PRSV-W CP probe. An equal amount of RNA (30 µg) was loaded in each lane. M: 21 and 25 nt long oligonucleotides used as size markers.

infection. The expression of TMV CP in transgenic tobacco was the first demonstration of genetically engineered resistance to a plant virus (Powell-Abel *et al*., 1986). Transgenic tobacco expressing the *cp* gene (Golemboski *et al*., 1990) of CMV showed strong resistance to virus infection. In melon, transformation using the *cp* genes of cucumber mosaic virus white leaf strain (CMV-WL), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV) showed that only transgenic plants expressed the CP were resistant to viruses (Gonsalves *et a*l., 1994, Fuchs *et al*., 1997). However, the expression of CP of rice yellow mottle virus (RYMV) enhanced virus infection in transgenic plants while the expression of antisense CP and non translatable CP mRNA exhibited a delay in symptom development (Kouassi *et al*, 2006). In our experiment, only one transgenic line containing the full length *cp* gene was infected by PRSV-W. The enhancement of virus infection in this transgenic line was not observed.

In addition to the high efficiency for generating transgenic plants resistant to a viral pathogen, the RNAi-mediated resistance has advantages for environmental biosafety over the CP mediated resistance. Several reports have shown that CPs expressed by transgenic plants can partially or completely encapsidate the genome of challenge RNA viruses (Lecoq *et al*., 1993; Farinelli *et al*., 1992; Hammond and Dienelt, 1997). Modified and novel viruses can develop through heterologous encapsidation and recombination. As there is no virus CP production in transgenic plants produced by RNAi mechanism, the potential risks of heterologous encapsidation and recombination of virus are diminished.

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References

Bernadac, A., Latché, A., Roustan, J. P., Bouzayen, M. and Pech, J. C. (2002) Fruit and vegetable biotechnology; in *Fruit and Vegetable Biotechnology*, Valpuesta, V. (ed.), pp. 257-261, Woodhead Publishing Limited, England.

Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Chen, Y-K., Lohuis, D., Goldbach, R. and Prins, M. (2004) High frequency induction of RNA-mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs. *Mol. Breeding* **14**, 215-226.

Dong, J. Z., Jia, Y. S. R. and Chua, N. H. (1991) Transformation of melon (*Cucumis melo* L.) and expression from the cauliflower mosaic virus 35S promoter in transgenic melon plants. *Biotechnology* **9**, 858-863.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. *Nature* **411**, 494-498.

Fang, G. and Gourmet, R. (1990) *Agrobacterium tumefacienes* mediated transformation and regeneration of muskmelon plants. *Plant Cell Rep.* **9**, 160-164.

Farinelli, L., Malnoe, P. and Collet, G. F. (1992) Heterologous encapsidation of potato virus Y strain O (PVYO), with the transgenic coat protein of PVY strain N (PVYN) in *Solanum tuberosum* cv. Bintje. *Biotechnology* **10**, 1020-1025.

Fassuliotis, G. and Nelson, B. V. (1992) Regeneration of tetraploid muskmelons from cotyledons and their morphological differences from two diploid muskmelon genotypes. *J. Am. Soc. Hortic. Sci.* **1175**, 863-866.

Fuchs, M., McFerson, J. R., Tricoli, D. M., McMaster, J. R., Deng, R. Z., Boeshore, M. L., Reynolds, J. F., Russell, P. F., Quemada, H. D. and Gonsalves, D. (1997) Cantaloupe line CZW-30 containing coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus-2 is resistant to these three viruses in the field. *Mol. Breeding* **3**, 279-290.

Gaba, V., Elman, E., Perl-Treves, R. and Gray, D. J. (1996) A theoretical investigation of the genetic variability in the ability of *Agrobacterium* to transform *Cucumis melo* L. in Gomez-Guillamon, M.L., Soria, C., Cuartero, J., Tores, J.A., Fernandez-Munoz, R., eds. Cucurbits Towards 2000, pp. 172-178, Proc. 6th Eucarpia Meeting on Cucurbit Genetics and Breeding, Malaga, Spain.

Gaba, V., Less, H. and Antigus, Y. (1992) Transformation of melon by particle acceleration. *Supplement of Plant Physiol.* **99**, 137.

Gaba, V., Zelcer, A. and Gal-On, A. (2004) Cucurbit biotechnology-the importance of virus resistance. *In Vitro Cell. Dev. Biol. Plant* **40**, 346-358.

Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. and Wilson, T. M. A. (1987) A comparison of eukaryotic viral 5'-leader sequences as enhancers of mRNA expression *in vitro*. *Nucleic Acids Res.* **15**, 8693-8711.

Golemboski, D., Lomonossoff, G. and Zaitlin, M. (1990) Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. *Proc. Natl. Acad. Sci. USA* **87**, 6311-6315.

Gonsalves, D., Xue, B., Yepes, M., Fuchs, M., Ling, K., Namba, S., Chee, P. and Slightom, J. L. (1994) Transferring cucumber mosaic virus-white leaf strain coat protein gene into *Cucumis melo* L. and evaluating transgenic plants for protection against infections. *J. Am. Soc. Hortic. Sci.* **119**, 345-355.

Guillemaut, P. and Marechal-Drouard, L. (1992) Isolation of plant DNA: A fast, inexpensive and reliable method. *Plant Mol. Biol. Rep.* **10**, 60-65.

Hamilton, A. J. and Baulcombe, D. C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952.

Hammond, J. and Dienelt, M. M. (1997) Encapsidation of potyviral RNA in various forms of transgene coat protein is not correlated with resistance in transgenic plants. *Mol. Plant Microbe Interact.* **10**, 1023-1027.

Hofgen, R. and Willmitzer, L. (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res.* **16**, 9877.

Jan, F.-J., Fagoaga, C., Pang, S-Z. and Gonsalves, D. (2000) A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. *J. Gen. Virol.* **81**, 235-242.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

Kouassi, N. K., Chen, L., Sire, C., Bangratz-Reyser, M., Beachy, R. N., Fauquet, C. M. and Brugidou C. (2006) Expression of rice yellow mottle virus coat protein enhances virus infection in transgenic plants. *Arch. Virol.* **151**, 2111-2122.

Lecoq, H., Ravelonandro, M., Wipf-Scheibel, C., Monsion, M., Raccah, B. and Dunez, J. (1993) Aphid transmission of a non-aphid-transmissible strain of zucchini yellow mosaic potyvirus from transgenic plants expressing the capsid protein of plum pox potyvirus. *Mol. Plant Microbe Interact* **6**, 403-406.

Mazier, M., German-Retana, S., Flamain, F., Dubois, V., Botton, E., Sarnette, V., Gall, O. L., Candresse, T. and Maisonneuve, B. (2004) A simple and efficient method for testing Lettuce mosaic virus resistance in *in vitro* cultivated lettuce. *J. Virol. Methods* **116**, 123-131.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum* **15**, 473-497.

Pang, S.-Z., Jan, F.-J. and Gonsalves, D. (1997) Non target DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants. *Proc. Natl. Acad. Sci. USA* **94**, 8261-8266.

Powell-Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T. and Beachy, R. N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.

Russo, P. and Slack, S. A. (1998) Tissue culture methods for the screening and analysis of putative virus-resistant transgenic potato plants. *Virology* **88**, 437-441.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, USA.

Sanford, J. C. and Johnston, S. A. (1985) The concept of parasite-derived resistance-deriving resistance genes from the parasite's own genome. *J. Theor. Biol.* **113**, 395-405.

Smith, N. A., Singh, S. P., Wang, M-B., Stoutjesdijk, P. A., Green, A. G. and Waterhouse, P. M. (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320.

Szittya, G., Molnar, A., Silhavy, D., Hornyik, C. and Burgyan, J. (2002) Short defective interfering RNAs of tombusviruses are not targeted but trigger posttranscriptional gene silencing against their helper virus. *Plant Cell* **14**, 359-372.

Tepfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H. (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res.* **15**, 5890.

Vaucheret, H., Beclin, C. and Fagard, M. (2001) Post-transcriptional gene silencing in plants. *J. Cell Sci.* **114**, 3083-3091.

Waterhouse, P. M. and Helliwell, C. A. (2003) Exploring plant genomes by RNA-induced gene silencing. *Nat. Rev. Genet.* **4**, 29-38.

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M-B., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D., Stoutjesdijk, P. A., Robinson, S. P., Gleave, A. P., Green, A. G. and Waterhouse, P. M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581-590.

Zamore, P., Tuschl, T., Sharp, P. and Bartel, D. (2000) RNAi: double stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.