J. Agr. Sci. Tech. (2012) Vol. 14: 1105-1120

A Dual Coat Protein Construct Establishes Resistance to Passionfruit Woodiness and *Cucumber Mosaic Viruses*

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ABSTRACT

There is a high degree (>95%) of intraspecies similarity in the coat protein (CP) amino acid sequences within *Passionfruit woodiness virus* (PWV) and *Cucumber mosaic virus* (CMV), both infecting passionfruit vine in New South Wales. On this basis, a dual transgene containing the translatable cDNAs coding for the CPs of PWV and CMV was constructed in the binary vector pBI121 and used for transformation of *Nicotiana benthamiana*, a susceptible host to both viruses. The transformation was achieved by co-cultivation of the agrobacteria with the leaf disks prepared from the surface- sterilized leaves. Five transgenic lines including 1-1, 1-5, 1-7, 1-12 and 1-24 were regenerated. Insertion and transcription of the dual construct were confirmed, however, only the CMV CP was feasibly detectable by DAS-ELISA in the lines. Low level accumulation of CMV and/or PWV was evident in the lines. In the initail challenge trial where 1:10 dilution of plant sap was used, a 5-day delay in symptom was generally shown. Inoculations with 1:100 plant sap also gave similar results as with 1:10 dilution. Lines 1-5 and 1-12, which were inoculated with 1:1000 dilution of sap, remained uninfected by CMV till 27 dpi, whereas with PWV, 1-12 became infected by 11 dpi. Four cuttings of line 1-12 reacted diffferently to the challenge inoculations i.e. three of them resisted PWV, whereas two of them were susceptible to CMV. Since PWV CP was not detectable in the transgenic lines but evidence of resistance to PWV was found in them, this was suggestive of an RNA silencing mechnaism involved in the resistance. Because the CMV CP was detectable in the transgenic lines, this suggested requirement for the CP expression in the resistance . The resistance, or apparent immunity, was manifested by an apparent delay in symptom expression and accumulation of relatively low levels of the viruses.

Keywords: Coat protein, *Cucumber mosaic virus*, Dual construct, Passionfruit woodiness virus.

INTRODUCTION

Passionfruit has long been threatened by the woodiness disease in Australia. The cause of the disease remained unknown until 1964 when, in Queensland, it was attributed to a distinct unidentified virus with flexuous rod-shaped particles about 670 nm in length, named as *Passionfruit woodiness virus* (PWV) which was placed in the potyvirus group (Taylor and Kimble, 1964). An accreditation scheme was established (Greber, 1966) and was in operation for many years to protect the passion vine. According to the scheme, the commercial hybrids of passionfruit, which contained mild strains of PWV, were released to passionfruit growers. Some of these mild-strain carrying hybrids (3-1 and 23-E) were still making up the major fresh fruit and processing varieties grown in New South Wales (NSW) by 1981. However, a severe strain overcame the mild strain and destroyed the protection. The variety 3-1 was so badly affected that it was lost from South East Queensland. A scionwood

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[DOR: 20.1001.1.16807073.2012.14.5.7.5]

scheme was started in 1981 to save the hybrids against attack by the severe strain. According to this scheme, the best individual vines in terms of productivity, trueness to type, and freedom from visual symptoms of the severe strain of PWV were selected and distributed to the growers (Peasley and Fitzell, 1981). An outbreak of "tip necrosis" disease devastated the passionfruit vines on the north coast of NSW and the investigation by Pares *et al*. (1985) revealed the involvement of *cucumber mosaic cucumovirus* (CMV) in a synergistic action with PWV, which killed the plant quickly while neither of the viruses alone was able to cause such a severe disease.

Developments in application of recombinant DNA technology to produce virus resistant transgenic plants (Dasgupta *et al*., 2003; Gallitelli and Accotto, 2001; Powell- Abel *et al*., 1986) have brought about new hopes to protect the Australian passionfruit from infection by the two viruses. PWV and CMV belong to two different genera of plant viruses: Potyvirus and Cucumovirus, respectively. Potyviruses have a single-stranded, positive-sense RNA genome of about 10 kilobases (kb) with a covalently attached virus-encoded protein at the 5′ end, and a 3′ poly-A tail. The genome is translated as a single polypeptide that is post-translationally processed by proteolysis into at least eight functional proteins (Dougherty and Carrington, 1988; Reichmann *et al*., 1992). The coat protein (CP) is the most studied part of the potyviruses. The CP of three strains of PWV described as tip blight (PWV-TB), severe (PWV-S) and mild strain (PWV-M) was estimated to be 275 amino acid residues (Shukla *et al*., 1988). PWV strain *K* (PWV-K), a severe isolate from Queensland, had 85-86% CP sequence similarity to the three strains (Gough and Shukla, 1992). They determined 1506 bp from the 3′ end of PWV-K which covers nucleotides at the 3′ untranslated region, the whole CP region (837 nucleotides, including a stop codon at the 3′ end) and 422 nucleotides from the 3′ end of the region coding for replicase protein (RP). Our previous results revealed that the PWV isolates from NSW had over 95% similarity to the strain K (Sokhandan *et al*., 1997).

CMV is the type species of the genus *Cucumovirus* that belongs to the family Bromoviridae. CMV has a divided genome consisting of three positive-sense single-stranded RNAs and a subgenomic RNA 4, which is generated by transcription from RNA 3 and acts as messenger RNA for the viral CP.

Protection conferred by a transgene against viruses that do not belong to the same group as the source virus of the transgene is weak or non-existent (Anderson *et al*., 1989). Therefore, it would not be expected that the CP transgene from either PWV or CMV would protect the plant against the other virus. However, production of transgenic plants transformed with a dual construct derived from two different viruses has been promising in establishing protection against the two viruses (Lawson *et al*. 1990; Bazzini *et al*., 2006). The aim of this study was to produce transgenic plants transformed with a construct composed of the CP cDNA of CMV and of PWV. At the time this research was carried out, regeneration of passionfruit from transformed callus was yet to be optimized so that the model plant *N. benthamiana* was subjected to the study.

MATERIALS AND METHODS

Designing CMV+PWV CP cDNA Construct

A dual construct containing CMV CP cDNA followed by PWV CP cDNA was designed with no stop codon between the cDNAs. The same PWV and CMV CP primers, as in our previous study (Sokhandan *et al*., 1997), with some modifications at CMV CPR and PWV CPF primers, were used (Table 1). A unique *Nar* I (GGCGCC) restriction site was

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

Table 1. Primers and their sequences.

Primer [a]	Sequence (5′-3′) [b]	Gene
CMV CPF	*5′ctcgaattcggatccGCTTCTCCGCGAG3′*	CMV CP
CMV CPR	*5′aaacacacggcgccAGTCGGGAGCATCCGTG3′*	CMV CP
PWV CPF	*5′ctgttggcgccatgTCTGGCAAAGAT3′*	PWV CP
PWV CPR	*5′gaattcgagctcTTACTGCACAGGCCCCAT3′*	PWV CP
npt-II F	*5′TCTCACCTTGCTCCTGCC3′*	*npt*-II gene
npt-II R	*5′AGGCGATAGAAGGCGATGC3′*	*npt*-II gene
non-tDNA F	*5′CGCTCTTTTCTCTTAGGTTTA3′*	non-tDNA RB
npt-II 5′	*5′ GTCATAGCCGAATAGCCTC3′*	npt-II 5′

[a] Primers npt-II F (forward), npt-II R (reverse), non-tDNA RB forward and npt-II 5′ (Worrall, 1998) npt-II 5′ was used as the reverse primer
[b] Engineered sequences are represented in lowercase and *Nar* I site is underlined.

incorporated in the CMV CPR and PWV CPF to facilitate joining of the cDNAs.

Amplification and Cloning of the CP cDNAs

CMV and PWV CP cDNAs were amplified and separately cloned into pGEM-T vector (Promega) as descibed elsewhere (Sokhandan *et al*., 1997). Colony PCR screening (Saris *et al*., 1990) of the resulting white colonies on LB (Luria Betani) plates containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-thiogalacto-pyranoside) was also done according to Sokhandan *et al*. (1997). Restriction analyses of the recombinant plasmids *pGEMPWVCP* or *pGEMCMVCP* were done with *Apa* I/ *Nar* I or *Nar* I/ *Sac* I, respectively, before subjecting them to sequencing with M13 F and M13 R primers.

Joining the CP cDNAs and Cloning

Apa I/ *Nar* I double digestion of *pGEMCMVCP* released the CMV CP cDNA and of pGEMPWVCP made it linearized because therin these sites are a few bases apart. The CMV CP cDNA and linearized *pGEMPWVCP* were purified from gel by a combination of the Gelase system (Epicentre Technologies, Madison, USA) and Amicon "Microcons" devices and ligated by the use of Promega T_4DNA ligase kit. *E. coli* JM109 was transformed with the ligated plasmid (pGEMCMPWCP) and the resultant colonies were selected and screened by PCR as described above before sequencing by M13 F, M13 R, CMV CPF, CMV CPR, PWV CPF, PWV CPR and a forward internal CMV CP primer corresponding to nucleotides close to the *Nar* I joining site. The nucleotide data were compared with the original CP cDNAs sequences to confirm correctness of the polymerization by *Taq* DNA polymerase cand loning.

Cloning into pBI121

*Bam*HI$_+$ *Sac*I digests of both pBI121 (ClonetTech, USA) and *pGEMCMPWCP* were fractionated on 1% LMP (low melting point) agarose and recovered, then were ligated to each other to prepare *pBICMPWCP* (Figure 1) that was used to transform *E. coli* JM109. The resultant white colonies were screened by PCR with CMV CPF and PWV CPR primers.

Transformation of *Agrobacterium* with *pBICMPWCP*

40 µl of competent *Agrobacterium tumefaciens* LBA4404 (Life Technology) cells was transferred into a sterile 1.5 ml

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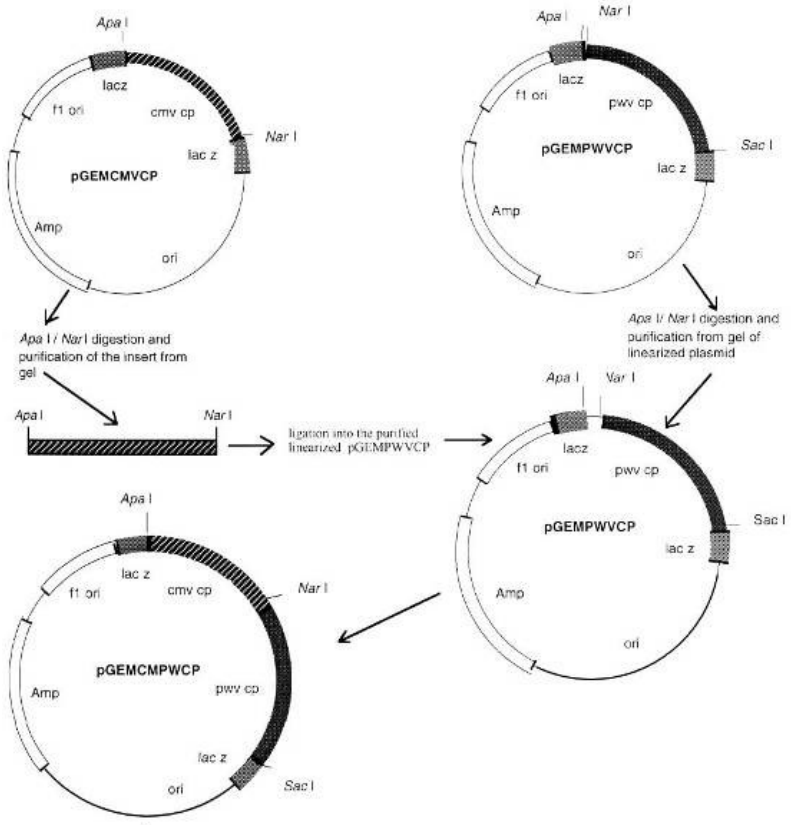

Figure 1. Ligating CMV and PWV CP cDNAs. Note that pGEMCMVCP and pGEMPWVCP are pGEM-T vectors carrying amplified CMV and PWV CP cDNA, respectively.

tube and 1µg of pBICMPWCP was added and mixed by pumping with the pipette. Then, the mixture was transferred into a disposable Pulser Cuvette (Bio- Rad, USA) and placed in the Gene Pulser before electroporation was carried out at standard settings (2.5 Kv, 200 Ω and 25 µF) for about 3.8 sec. Then, the mixture was transferred into a 12 ml Falcon tube and placed in a 28°C shaking incubator at 200 rpm for 2 hours. A 200 µl aliquot of the culture was spread on a MG L^{-1} (Cangelosi *et al*., 1991) plate containing kanamycin (km) and rifampicin (rif) each at 50 mg ml^{-1} followed by incubation at 28°C for 48 to 72 hours. The resulting transformants were screened by PCR by the use of CMV CPF and PWV CPR as described previously. The initial denaturation at 94°C for 2 minutes was followed by 5 cycles of 94°C for 30 seconds, 40°C for 30 seconds, and 72°C for 2 minutes. Then, 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes were applied before completing The polymerization at 72°C for 7 minutes.

Transformation of *N. benthamiana*

Three *A. tumefaciens* LBA4404 lines containing *pBICMPWCP*, *pBI121* (control) or disarmed Ti plasmid (control) were used. The first two lines were grown in 5 ml MG L^{-1} containing km and rif each at 50 µg ml^{-1}. The disarmed cell line was grown in the presence of rif only. The cultures were grown at 28°C in a shaking incubator at 250 rpm for 48 hours.

Leaf (explants) were prepared from young leaves of glasshouse- or aseptically- grown *N. benthamiana*. A solution containing 1% sodium hypochlorite and 0.1% Tween-20 was used for surface sterilizaion of the leaves for 5, 7, 10 or 15 minutes; consequently, sterilization

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for 5 minutes appeared to be satisfactory. The leaves were rinsed with sterile water four times and placed on a sterile Whatman paper to blot the excess water. Leaf strips, ~ 0.5×1 cm were cut out from the leaves and placed in 1:50 dilution of overnight grown agrobacterial suspensions for 5 minutes with occasional shaking and followed by blotting on a sterile filter paper to remove the excess bacteria. Then, the explants were placed on the shooting medium containing MS salts and vitamins (Murashige and Skoog, 1962), 1 mg l^{-1} BAP (benzyl amino purine) and 0.1 mg l^{-1} NAA (nicotine aceic acid) in 9 cm Petri dishes (10 to 15 strips per plate) and incubated at 25°C under 0.09 Watts m^{-2} light intensity for 3-4 days before they were transferred onto the shooting medium containing km and cefotaxime (100 µg ml^{-1} each). After 3-4 weeks, when the shoots were about 1.5 cm they were excised from the explants and transferred onto the rooting medium containing MS salts and vitamins, 1 mg l^{-1} IBA (indole butrytic acid), km and cefotaxime (100 µg ml^{-1} each) in tissue culture jars (5 shoots per jar) and incubated at 25°C under the same light condition as described above. In the preparation of the media, pH was adjusted to 5.75 prior to addition of 0.2% Phytagel (Sigma) and autoclaving at 121°C for 20 minutes.

Km-resistant plants were removed from the jars, cleaned off the medium under tap water, and planted in 9 cm pots containing a 50:50 mixture of sterile perlite and vermiculite. The pots were transferred into a mist chamber under low light at 25°C for about 2 weeks, then, transferred into a light room with 16 hours photoperiod at 25°C.

PCR Analysis of Transformed Plants

Genomic DNA was extracted from each transformed line according to Edwards *et al*. (1991) with the modifications suggested by Yu and Paul (1994). DNA extracts from two transformed lines grown on the medium containing only *Cx* were used as the controls. Concentrations of the DNA samples were determined in a spectrophotometer. It was crucial to confirm that *Agrobacterium* was removed from the plants before further analysis. Accordingly, PCR with a primer specific to the binary vector from beyond the T-DNA right border (non-tDNA F) (Worrall, 1998) and another primer binding to the *npt*-II region (npt-II 5′) was done on the genomic DNA samples. Two pmol of each primer and 5 µl aliquots of genomic DNA samples (total volume 100 µl) were used in the reactions. In the positive control, *pBICMPWCP* was used as the template. In the two negative control reaction mixes, either genomic DNA from a wild type *N. benthamiana* plant or no template was used.

Later, the plants were checked with two sets of primers based on the dual construct (CMV CPF and PWV CPR) or *npt*-II, respectively (Table 1). PCR reactions were prepared in 40 µl containing 2 pmol of each primer and 5-8 µl of DNA. PCR on *pBICMPWCP* was used as the positive control. As a negative control, the reaction was carried out on the genomic DNA from a wild-type *N. benthamiana*. "Hot-start" PCR (Chou *et al*., 1992) was performed such that the reactions lacking *Taq* DNA polymerase were heated to 80 °C in the thermal cycler for 5 minutes before aliquoting the enzyme into the reactions at 80 °C. Then, an initial denaturation at 95 °C for 2 minutes was followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 2 minutes and finally the polymerization was completed at 72 °C for 7 minutes. Similarly, PCR with *npt*-II F and *npt*-II R primers was performed. Representative PCR products were cloned and sequenced as described previously to confirm that the fragments amplified by the construct-specific primers had resulted from the transgene.

Analysis of Transgene Transcription

Total RNA was extracted from 60 mg leaf tissue of the transformed *N. benthamiana* plants by the use of Promega RNAgents total RNA isolation kit according to the

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manufacturer's protocol. First, "Hot-start" PCR with CMV CPF and PWV CPR was carried out on 5 µl (out of 100 µl) of the RNA samples as described above to know if the samples had DNA contamination. Then, 50 µl out of 100 µl total RNA extracts from the samples having DNA contamination was digested with Promega RQ I RNase-free DNase according to the manufacturer's protocol. An amplified PWV CP cDNA and total RNA from a PWV-infected *N. benthamiana* were used as two controls to check the efficacy of the DNase and its possible degrading effect on RNA, respectively. A digestion mix for each sample was set up in 100 µl containing 1×RQ I DNase buffer, 10 mM DTT, 2 units of Promega RQ I RNase-free DNase and 100 units of rRNasin Ribonuclease inhibitor. The digests were purified through phenol: chloroform (1:1) extraction and precipitation with 2 and 0.1 volumes of ethanol and 3 M sodium acetae, respectively.

Reverse transcription was performed with 2.5 pmol of oligo $d(T)_{16}$ in 10 a µl reaction mix for each sample. The reverse transcription was done as described elsewhere (Sokhandan *et al*., 1997). PCR reaction mixes were prepared in 40 µl for each sample containing CMV CPF and PWV CPR. In the positive control, total RNA from a PWV- infected *N. benthamiana* was used as the template. In the negative control, total RNA from a healthy wild type *N. benthamiana* was used as the template. "Hot-start" PCR, as described above, was performed on the reverse-transcribed mixes.

Analysis of Transgene Expression

The indirect ELISA method (Clark *et al*., 1988) was applied using mouse anti-potyvirus IgG and rabbit anti-mouse alkaline phosphatase conjugate (Agdia, Elkhart, Indiana) to detect the expression of PWV CP in the transformed *N. benthamiana* plants. For the detection of CMV CP expression in the transformed plants, CMV polyclonal IgG and conjugate from Sanofi (Phyto-diagnostics, Marnes-La-Coquette-France) were used in double-antibody sandwich ELISA (DAS-ELISA) (Clark and Adams, 1977) according to the manufacturer's protocol.

In the ELISA on the transgenic plants 1: 5 dilutions of sap, 1: 200 dilutions of the IgG and conjugate were used and the controls were included as in the preliminary tests. Extraction was done in ceramic mortar or a 1.5 ml microfuge tube. Three leaf discs (about 70 mg) were pinched out from leaves of each line with the lid of a 1.5 ml Eppendorf tube containing 0.450 ml extraction buffer. The leaf discs were ground by a polypropylene pestle fitting into the tube until a homogenous sap was obtained.

Assessment of Resistance in the Transgenic Plants

Because dual infections of passionfruit by PWV and CMV occur in the field (Pares *et al*., 1985; Sokhandan *et al*., 1997), most of the transgenic lines were simultaneously challenged with both viruses to know if the plants react similarly to both viruses. Passionfruit samples showing mosaic were obtained from the North Coast of New South Wales. The samples were checked for the presence of PWV and CMV by RT-PCRs with respective CP primers as described in Sokhandan *et al*. (1997). As a result, PWV was detected in one sample and CMV in another. CMV and PWV were propagated in *Nicotiana glutinosa* and *N. benthamiana*, respectively. R_0 regenerated and wild type *N. benthamiana* plants were challenged with 1:10, 1:100 or 1:1000 dilutions of PWV and/or CMV- infected sap. Mock-inoculated plants were included in the trials. The challenged plants were transferred into a growth chamber and the infection in the plants was monitored by ELISA on 1:100 or 1:1000 dilution of sap from the challenged plants using CMV and/or PWV reagents as described above.

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RESULTS

Integration of Transgene

PCR with non-tDNA F and *npt*-II 5′ primers on the genomic DNA from the putative transformed plants did not result in any amplification, indicating removal of the *Agrobacterium* from the plants. But, PCR on the genomic DNA samples using either construct- or *npt*-II-specific primers resulted in the amplification of the expected DNA fragments from the samples (Figure 2).

In most cases when PCR with construct-specific primers did not result in amplification but a 464 bp DNA fragment was amplified with *npt*-II-specific primers, PCR with the construct-specific primers and 8 µl (but not 5 µl) of genomic DNA in 40 µl reaction mix resulted in the amplification of the expected DNAs. PCR with construct-specific primers did not result in amplification from lines 1-15, 1-16and 1-21, but a 464 bp fragment was amplified when *npt*-II specific primers were used in the assay (Table 2). This raised the possibility that a deletion occurred in the transgene during its insertion into the plant chromosome in those lines.

There was only one line (1-2) among the 21 screened lines that did not give amplification with the construct- or *npt*-II-specific primers, and no DNA was amplified when an RT-PCR assay was carried out on this line (Table 2), suggesting that it was not transformed i.e. an escape. There was not any case where the transgene could be amplified but not the *npt*-II gene.

Transgene Transcription

RT-PCR with CMV CPF and PWV CPR on RQI DNase- treated total RNA samples from transformed plants resulted in the

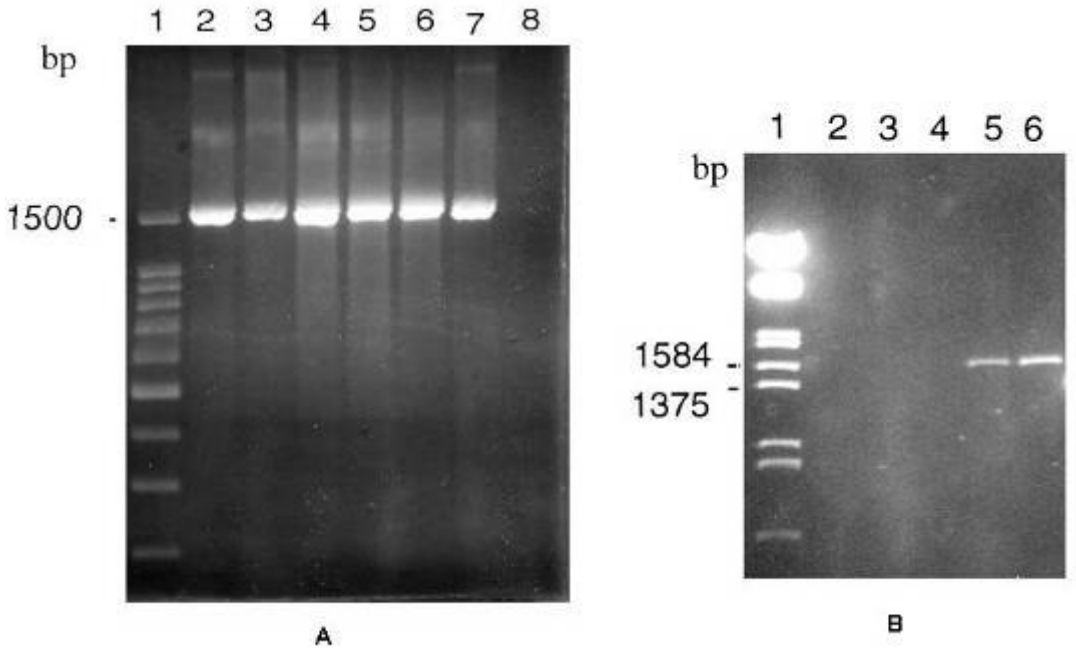

Figure 2. Agarose gels (1.5%) representing some of the PCR (A) and RT-PCR (B) products resulting from analyses of transgenic *Nicotiana benthamiana* plants. (A) lane 1: Promega 100 bp ladder marker DNA, lane 2- 6: products (1604 bp) from lines 1-3, 1-4, 1-5, 1-12 and 1-17, respectively, lane 7: PCR on pGEMCMPWCP (positive control), lane 8: PCR on a wild type plant (negative control); (B) lane 1: λ DNA *Eco*RI+ *Hind*III, lanes 2-4: RT-PCR on lines 1-2, 1-6 and 1-11 (no amplification), lanes 5- 6: lines 1-12 and 1-24, respectively.

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Table 2. Data resulting from analysis of *N. benthamiana* plants transgenic for CMV+PWV CP cDNA.

Line[a]	PCR[b]	PCR[c]	RT-PCR[b]	ELISA[d]	
				Anti-CMV	Anti-Poty
1-1	+	+	+	0.328 (+)	0.210 (?)
1-2	-	-	-	N/T	N/T
1-3	+		+	0.566 (+)	0.029 (-)
1-4	+	+	+	0.243 (?)	0.070 (?)
1-5	+	+	+	0.295 (?)	0.023 (-)
1-7	+	+	N/T	N/T	0.066 (?)
1-8	+	+	N/T	N/T	N/T
1-9	+	+	N/T	N/T	0.022 (-)
1-10	+	+	N/T	N/T	N/T
1-11	+	+	+	0.436 (+)	0.019 (-)
1-12	+	+	+	0.345 (+)	0.017 (-)
1-13	+	+	N/T	0.191	N/T
1-14	+	+	N/T	N/T	N/T
1-15	-	+	N/T	N/T	N/T
1-16	-	+	N/T	N/T	N/T
1-17	-	+	+	0.284 (?)	0.023 (-)
1-18	+	+	N/T	N/T	N/T
1-20	+	+	N/T	N/T	N/T
1-21	-	+	N/T	N/T	N/T
1-24	+	+	+	0.176 (-)	0.024 (-)
WT	-	-		**0.164**	**0.019**
WT-PWV					0.903
WT-CMV				0.707	N/T

[a] Lines resulting from transformation with the dual construct (CMV+PWV CP cDNA); WT: Wild type *N. benthamiana*; WT-PWV: Wild type *N. benthamiana* inoculated with PWV; WT-CMV: Wild type *N. benthamiana* inoculated with CMV.
[b] PCR with CMV CPF and PWV CPRr
[c] PCR with *npt*-II F and *npt*-II R
[d] N/T: ELISA could not be performed because the transgenic line (clone) did not survive the greenhouse likely because of root fungal infection. The threshold was set at twice average absorbance value of wells treated with healthy sap (WT), or average absorbance value of such wells plus three times standard deviation between them. Samples, negative by the former but positive with the latter threshold, are shown with (?). (+) or (-) stands for positive or negative absorbance value, respectively.

amplification of 1,604 bp fragment from 8 lines. RT-PCR with PWV CP primers on the RQI DNase- treated total RNA preparation from a PWV-infected *N. benthamiana* (positive control) resulted in the amplification of a 860 bp DNA fragment, suggesting that the treatment did not damage the RNA preparations. RT-PCR on nine transformed lines resulted in the amplification of a 1604 bp DNA fragment from 8 of them (Table 2, Figure 2).

Transgene Expression

ELISA, using the respective reagents, detected the CP of CMV and/or PWV in some of the transgenic lines carrying the dual construct. Expression of CMV CP was detected in lines 1-1, 1-3, 1-11 and 1-12 based on twice average absorbance value of the negative control (wild type plant) as the threshold. However, the expression in lines 1-4, 1-5 and 1-17 was detectable only if the

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

threshold was set to average absorbance value of the negative control plus three times standard deviation between them. In case of PWV CP, because the absorbance value of the negative control was almost zero (0.019) and the color change was not observed by eye, it was difficult to determine whether or not the CP was expressing, although the highest absorbance value was associated with line 1-1 (0.210) (Table 2).

Analysis of Resistance

A total of 34 transgenic R_0 plants were generated and 25 plants including 5 lines (1-1, 1-5, 1-12, 1-7 and 1-24) survived acclimatization. As a result, from an initial trial wherein the plants were challenged with a low dilution (1:10) of the infected sap, cuttings of line 1-12 delayed the infection for 5 days. Although ELISA 11 dpi (days post-inoculation) indicated that all the transgenic lines were infected, the accumulation of CMV and/or PWV was at a lower level in comparison to the wild type controls. ELISA also showed that the 3 cuttings of line 1-12 had no PWV infection at least 11 dpi. Also, 2 out of 4 cuttings of line 1-12 were infected at 11 dpi as revealed by ELISA indicating a variation in the protection among the cuttings. There was no symptom on the mock-inoculated wild type plants and no infection was detectable by ELISA in them (Table 3).

Similar results were obtained when 1:100 dilutions of infected sap was used in the challenge. ELISA 11 and 18 dpi showed that the transgenic lines generally accumulated low level of PWV and /or CMV in comparison to the wild type controls (Table 4). Line 1-12 that had shown no infection 11 dpi when challenged with 1:10 dilution of sap remained uninfected for at least 11 dpi after challenge with 1:100 infected sap dilution. A cutting of line 1-12, when challenged with 1:100 dilution of a mixture of the two viruses, showed infection within

Table 3. Evaluation of resistance in transgenic *Nicontiana benthamiana* plants using higher dilution (1:10) of inoculum.

Line [a]	Challenge [b]	Anti-poty [c]	Anti-CMV ($A_{405\ nm}$) [d]		Symptom [f]
		($A_{405\ nm}$) 11 dpi	11 dpi	18 dpi	at 11 dpi
WT -1	PWV+CMV	0.697	0.435	0.348	many LCs
WT-2	PWV+CMV	0.519	0.484	0.174	many LCs
1-1	PWV+CMV	0.274	0.470	0.146	LCs
1-12-1	PWV+CMV	0.004	0.021	0.315	-
1-12-2	PWV+CMV	0.009	0.026	0.115	-
1-12-3	PWV+CMV	0.558	0.442	0.341	LCs
1-12-4	PWV+CMV	0.020	0.217	0.442	-
1-17	PWV+CMV	0.412	0.024	0.230	-
WT-3	PWV	0.536	N/A [e]	N/A	many LCs
WT-4	PWV	0.505	N/A	N/A	many LCs
WT	-	0.040	0.019	0.010	-

[a] WT: Wild type.
[b] The type of inoculum used in the challenge either PWV or mixture of PWV and CMV (PWV+CMV); -: not challenged.
[c] ELISA with anti-poty reagents 11 days after challenge: The values represent the mean absorbance ($A_{405\ nm}$) of 3 replicate wells. The threshold was set at twice average absorbance value of wells treated with healthy sap (WT).
[d] ELISA with CMV reagents 11 and 18 days after the challenge- the values represent the mean absorbance ($A_{405\ nm}$) of 3 replicate wells. The threshold was set at twice average absorbance value of wells treated with healthy sap (WT).
[e] N/A: Not applicable
[f] Symptoms 11 days after the challenge; LC: Leaf curling; -: no symptoms.

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Table 4. Evaluation of resistance in transgenic *N. benthamiana* plants using higher dilution (1:100 and/or 1:1000) of inoculum.

Line [a]	Challenge	Sap [b]	Reagents	ELISA [c] at 11 dpi	ELISA at 18 dpi	Symptom at 22 dpi
WT-1	PWV	1:100	anti-poty	0.897	1.325	stunting, yellowing
1-12	PWV	1:100	anti-poty	0.016	1.269	some leaf curlings
WT-2	PWV+CMV	1:100	anti-poty	0.721	1.154	mosaic, leaf curling
1-12	PWV+CMV	1:100	anti-poty	0.346	1.052	No symptoms
1-24	PWV+CMV	1:100	anti-poty	0.076	1.249	No symptoms
WT	-	1:100	anti-poty	0.029	0.009	No symptoms
WT-3	PWV+CMV	1:1000	anti-poty	0.449	1.018	stunting, yellowing
1-5	PWV+CMV	1:1000	anti-poty	0.027	0.012	No symptoms
1-12	PWV+CMV	1:1000	anti-poty	0.703	1.134	leaf curlings
WT-3	PWV+CMV	1:1000	anti-CMV	0.523	0.523	stunting, yellowing
1-12	PWV+CMV	1:1000	anti-CMV	0.031	0.031	No symptoms
1-5	PWV+CMV	1:1000	anti-CMV	0.023	0.035	leaf curlings
WT-4	-	1:100	anti-CMV	0.032	0.023	No symptoms

[a] WT: Wild type.
[b] Dilution of infected plant sap used as inoculum.
[c] The threshold was set to twice average absorbance value of wells treated with healthy sap (WT).

11 dpi. However, accumulation of PWV and CMV in this line was less than half of the level in the wild type controls. Line 1-24 did not show infection 11 dpi but became infected in the following days, as a result, a positive ELISA reaction was obtained 28 dpi.

A plant of line 1-5 and 1-12 challenged with 1:1000 dilution of PWV and CMV remained uninfected by CMV even 27 dpi but line 1-12 became infected with PWV within 11 dpi (Figure 3). However, accumulation of the virus in line 1-12 was significantly less than in the wild type controls. The difference in the reaction of the two lines against PWV could not be correlated with the PWV CP expression because the CP was undetectable in both lines.

DISCUSSION

In this study, a dual construct containing CP cDNAs of both CMV and PWV was prepared and inserted into the genome of the model plant *N. benthamiana* that ended up in manifestations of resistance in the R_0 transgenic lines.

PCR screening of *A. tumefaciens* colonies (transformed the construct) by direct suspension of the colony in the PCR mix (Saris *et al*., 1990) failed, whereas such a PCR method was productive in screening *E. coli* colonies. This is possibly due to the presence of inhibitors in the *Agrobacterium* i.e. by suspending the colonies in water, the inhibitor was diluted to a level that had no inhibitory effect on the PCR.

When leaves of glasshouse-grown *N. benthamiana* plants were surface-sterilized with sodium hypochlorite for 15 minutes, all the explants died within a few days. A trial that was set up to determine an optimum period of time for the surface sterilization indicated that even 5 minutes sterilization was sufficient. It is important to recognize that the sterilization period may differ according to the host species and the optimal period should be determined when working with a new species (e.g., Oyebanji *et al*., 2009)

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

Figure 3. Wild type (right) and transgenic (line 1-5) for CMV+PWV CP cDNA (left) *Nicotiana benthamiana* plants challenged with a 1:1000 dilution of CMV- and PWV- infected sap. The wild type control became infected (as revealed by ELISA) at 11 dpi and developed symptoms subsequently, including chlorosis and stunting. The transgenic plant did not develop any symptoms and no infection was detected by ELISA.

The transformation of *N. benthamiana* was achieved through optimization. The shooting medium initially contained MS salt (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al*., 1968), 3 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, which gave an excessive enlargement of the explants and, as a result, the explants lost their contact with the medium at the edges. Shootings did not occur on such explants or occurred very late (after more than a month) and they did not root on the rooting medium that contained 1 mg l^{-1} IBA (indole butrytic acid). Soaking of the shoots in a concentrated solution of IBA (50 mg l^{-1}) did not promote the rooting either. A trial, set up to determine an optimum concentration of BAP in the medium, showed that 1 mg l^{-1} BAP in the shooting medium was sufficient for production of the shootings on the explants. Various plants require different quantities of the growth hormones for their optimal micro propagation and regeneration, although it seems that 1 mg l^{-1} BAP gives the best results with many plant species (e.g., Ather *et al.*, 2009).

In PCR analysis of the regenerated plants, incorporation of the construct-specific primers did not result in amplification from lines 1-15, 1- 16, 1-17 and 1-21, but a 464 bp fragment was amplified when *npt-II*-specific primers were used (Table 1). This raised the possibility that a deletion occurred in the transgene during its insertion into the plant chromosome in these lines. There was only one line (1-2) among the 21 screened lines that did not give amplification with the construct- or *npt-II*- specific primers, and no transgene transcript was detected when an RT-PCR assay was carried out on this line (Table 2), suggesting that it was not transformed i.e. an escape. Although growth on Km is a good indicator of a transformation event, there could be some plants among the population that escape the selection pressure of Km (Worrall, 1988). This could be due to the occurrence of shoots on the edges of explants where the contact with the selective medium might be

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

lost as the explants expand. To avoid the problem, the explants on the medium containing growth hormones should be observed regularly and the edges of explants that have lost contact with the medium should be folded back on the medium, or the explants should be excised. This was practiced in the current study.

The CMV CP was clearly detected in four lines (1-1, 1-3, 1-11 and 1-12), whereas with the PWV CP it was difficult to determine if the CP was expressing. This was because the absorbance value for the negative control was near zero (0.019) and, assuming the twice-average absorbance value of the negative control (wild type) as the threshold, then, lines 1-1, 1-4 and 1-7 would have been positive, i.e. they were expressing the CP. However, since the color change was not observed with eye, it was difficult to ascertain the expression. Therefore, one can conclude that even if these lines expressed the CP, the expression level was very low. The discrepancy of expression of the two CPs could be due to conformation of the expressed fused protein. The CP could not be detected probably because the PWV CP epitopes were not exposed. The other possibility could be RNA degradation, which may occur in transgenic plant expressing potyvirus CP gene after the transgenic RNA level reaches a threshold (Goldbach *et al*., 2003; Guo *et al*., 1998; Smith *et al*., 1995; Lindbo *et al*., 1993b). Although the PWV CP transcripts were detected by RT-PCR in most of the examined lines, it is possible that, by then, the RNA level was below the threshold, whereas examination of the expression was performed late after the RT-PCR analysis.

There was evidence of protection conferred against CMV and/or PWV in *N. benthamiana* plants transgenic for the dual construct. The protection was manifested by an apparent delay in the infection and accumulation of low levels of the viruses. The lines in which the infection was delayed and showed low levels of the viruses reacted similarly when challenged with various concentrations of infected sap. Resistance for low levels of challenge inoculum has been reported for potyviruses in transgenic plants. For example, there has been a delay in symptom expression in some lines of *N. benthamiana* plants transgenic for vanilla necrosis potyvirus (VNV) coat protein when inoculated with 10 mg l^{-1} VNV, but the plants were completely susceptible to more than 50 mg l^{-1} of the virus (Wang *et al*., 1997). Indeed, the first application of pathogen-derived resisance, which used TMV CP as the transgene, resulted in delay of symptom (Powell- Abel *et al*., 1986). Later on, delay in infection and accumulation of lower levels of respective challenge virus were reported in tobacco plants expressing CMV CP (Cuozzo *et al*., 1988) or *Pepper severe mosaic virus* (PSMV) CP (Rabinowicz *et al*., 1998). However, the mechanism in TMV CP expressing plants (Powell- Abel *et al*., 1986) seems to be different from that in our study because, in the case of TMV, it appeared that the mechanism is acting at disassembly stage as a later study revealed that inoculation with TMV RNA overcame the resistance (Nelson *et al*., 1987). Further studies have shown that the mechanism may act at different stages of virus infection cycle depending on the combination of transgene and invading virus (Dasgupta *et al*., 2003). They report that in resistance to TMV the mechanism acts at the virus disassembly stages and in the long-distance transport stage. According to these authors, in the case of *Alfalfa mosaic virus* (AlMV), it acts only at the disassembly stage, whereas in resistance to PVX it is involved at multiple stages including replication, cell-to-cell and systemic movement. Also, in tospoviruses, the stage affected is believed to be replication.

Also, it has been reported that the resistance is mediated by the transgene RNA because a recovery phenomenon has been recored in the transgenic line (Rabinowicz *et al*., 1998). In our study, although we could not continue our work through R_1 generation, non detectability of the PWV CP in the transgenic lines and, at the same time,

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manifestation of resistance in them could be suggestive of an RNA-mediated mechanism.Similar reports on transgenic resistance to potyviruses have been made by others wherein they have pointed to the transgene silencing (Guo *et al*., 1998; Smith *et al*., 1995; Lindbo *et al*., 1993b). Posttranscriptional gene silencing (PTGS) is a natural phenomenon in plants to battle viruses; however, plant viruses have also evolved to use strategies to counterdefend the host plant defence system or, in other words, suppress the host gene silencing apparatus (Vance and Vaucheret, 2001). To that end, plant viruses use their own encoded proteins to carry out the counterdefense action; for example, HC-pro of potyvirus, 2b of CMV and P19 of tombusvirus (Li and Ding, 2006). Plant viruses can be the cause and target of PTGS so that certain viruses become silenced through PTGS and, as a result, infected plants recover from virus infection. One drawback for the resistance mediated by PTGS is its dependence on temperature; for instance, cassava geminivirus-induced RNA silencing is increased when temperaure is raised from 25 to 30ºC (Chellappan *et al*., 2005). Such a dependence may also be the case for other viruses.

In regard to resistance to CMV, since the CP was detected in the transgenic lines, which showed evidence of resistance, it may be conlcuded that such a resistance requires expression of the CP. This conclusion can be supporterd by previous reports necessitating expression of the CMV CP for the protection against the virus (Morroni *et al*., 2008; Kaniewski *et al*.,1998).

Not only different lines, but also cuttings of a transgenic lines may react differently to challenge inoculations as reported by others (Farinelli and Malonoë 1993). Here, it was evident that cuttings of line 1-12 reacted differenly to the challenge inoculations. Such variation in reaction to challenge inoculations has been reported in the cuttings of two R_0 tobacco lines transgenic for the PVY^N CP gene. Variation in the clones of a tobacco line transgenic for potyvirus, *Pepper severe mosaic virus* (PSMV) CP gene has also been reported (Rabinowicz *et al*., 1998). Here, three cuttings of line 1-12 developed no symptoms after the challenge inoculation whereas the cutting 1-12-3 showed leaf curles (Table 3). Therefore, these three cuttings and line 1-5 showed immunity against PWV or PWV+CMV. It seems that, as reported for other potyviruses (Lindbo *et al*., 1993a; Smith *et al*., 1994), the mechanism is RNA-mediated. The appearance of resistance in clones of line 1-12 might be explained by the model suggested by Smith *et al*. (1994). Accordingly, transgenic RNA accumulation above a critical threshold might induce an RNA degrading mechanism following viral amplification. Because line 1-12 transcribed the transgenic RNA (Figure 2, Table 2) but did not express the CP at least at a level detectable by ELISA, it is suggested that the protection has been RNA –mediated. In the susceptible clones, the transgene RNA level probably did not reach the threshold level to trigger RNA degradation, whereas resistance in the other clones of line 1-12 suggests that the threshold was achieved and the degradation was triggered.

In this research, for the first time in Australia, , an engineered resistance strategy against CMV and PWV was examined in the model plant that came out as promising because protection against the viruses was evident in the transgenic lines. The mechanism appeared to be the CP expression for CMV, but RNA-based for PWV; however, further analyses are required to ascertain the precise mechanism behind these protections. It seems obvious that the mechanism would not be the same for both viruses and each mechanism has its own advantages and drawbacks. Although PTGS is increasingly reported as more efficient, it may suffer from its dependence on temperature. The study should be extended to the next generations of the transgenic seeds accompanied by further molecular analyses of the transgene and challenge inoculation examinations. This

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

control strategy should also be adopted for passionfruit itself, which has been the ultimate goal of this study in order to save the Australian passionfruit from these two malicious viruses. Recent optimization of regeneration from transformed passionfruit explants should make this work feasible.

REFERENCES

1. Anderson, E. J., Stark, D. M., Nelson, R. S., Tumer, N. E. and Beachy, R. N. 1989. Transgenic Plants that Express the Coat Protein Gene of TMV or Almv Inferere with Disease Development of Non-related Viruses. *Phytopathol.,* **12:** 1284-1290.
2. Ather, A., Khan, S., Rehman, A. and Nazir, M. 2009. Optimization of the Protocols for Callus Induction, Regeneration and Acclimatization of Sugarcane cv. Thatta-10. *Pak. J. Bot.,* **41:** 815-820.
3. Bazzini, A. A., Asurmendi, S., Hopp, H. E. and Beachy, R. N. 2006. *Tobacco Mosaic Virus* (TMV) and *Potato Virus X* (PVX) Coat Proteins Confer Heterologous Interference to PVX and TMV Infection, Respectively. *J. Gen. Virol*., **87:** 1005–1012
4. Cangelosi, G. A., Best, E. A., Martinetti, G. and Nester, E. W. 1991. Genetic Analysis of Agrobacterium. *Methods Enzymol.,* **204:** 384-397.
5. Chellappan, P., Vanitharani, R., Ogbe, F. and Fauquet, C. M. 2005. Effect of Temperature on Geminivirus-induced RNA Silencing in Plants. *Plant Physiol*., **138:** 1828-1841.
6. Chou, Q., Russell, M., David, E. B., Jonathan, R. F. and Bloch, W. 1992. Prevention of Pre-PCR Mispriming and Primer Dimerization Improves Low-copy-number Amplifications. *Nucleic Acids Res.*, **20:** 1717-1723.
7. Clark, M. F., and Adams, A. N. 1977. Characteristics of Microplate Method of Enzyme-linked Immunosorbent Assay for the Detection of Plant Viruses. *J. Gen. Virol.,* **34:** 475-483.
8. Clark, M. F., Lister, R. M. and Bar-Joseph, M. 1988. ELISA Techniques. In: *"Methods for Plant Molecular Biology*", (Eds.): Weissbach, A. and Weissbach, H. Academic Press, San Diego, **32.**
9. Cuozzo, M, O'Connell, K., Kaniewski, W., Fang, R. X., Chau, N. H. and Tumer, N. E. .1988. Viral Protection in Transgenic Plants Expressing the *Cucumber Mosaic Virus* Coat Protein or Its Antisense RNA. *Bio. Tech.,* **6**: 549-557.
10. Dasgupta, I., Malathi, V. G. and Mukherjee, S. K. 2003. Genetic Engineering for Virus Resistance. *Curr. Sci.,* **84:** 341-354.
11. Dougherty, W. G. and Carrington, J. C. 1988. Expression and Function of Potyviral Gene Products. *Annu. Rev. Phytopathol.,* **26:** 123-143.
12. Edwards, K., Johnstone, C. and Thompson, C. 1991. A Simple and Rapid Method for the Preparation of Plant Genomic DNA For PCR Analysis. *Nucleic Acids Res.,* **19:** 1349.
13. Farinelli, L. and Malnoë, P. 1993. Coat Protein Gene-mediated Resistance to *Potato Virus Y* in Tobacco: Examination of the Resistance Mechanisms: Is the Transgenic Coat Protein Required for Protection? *MPMI,* **6:** 284-292.
14. Gallitelli, D. and Accotto, G. P. 2001. Virus-resistant Transgenic Plant: Potential Impact on the Fintess of Plant Viruses. *Euro. J. Plant Pathol.,* **83:** 3-9.
15. Gamborg, O. L., Miller, R. A. and Ojima, K. 1968. Nutrient Requirement of Suspension Cultures of Soybean Root Cells. *Exp. Cell res.,* **50:** 151-8.
16. Goldbach, R., Bucher, E. and Prins, M. 2003. Resistance Mechanisms to Plant Viruses: An Overview. *Virus Res.,* **92:** 207-212.
17. Gough, K. H. and Shukla, D. D. 1992. Major Sequence Variations in the N-Terminal Region of the Capsid Protein of a Severe Strain of Passionfruit Woodiness Potyvirus. *Arch. Virol.,* **124:** 389-396.
18. Guo, H. S., Cervera, M. T. and García, J. A. 1998. Plum Pox Potyvirus Resistance Associated to Transgene Silencing that Can Be Stabilized after Different Number of Plant Generations. *Gene,* **206:** 263-272.
19. Greber, R. S. 1966. Passion-fruit Woodiness Virus as the Cause of Passion Vine Tip Blight Disease. *Qld. J. Agric . Animal Sci.,* **23:** 533-538.
20. Kaniewski, W., Ilardi, V., Tomassoli, L., Mitsky, T., Layton, J. and Barba, M. 1999. Extreme Resistance to *Cucumber Mosaic Virus* (CMV) in Transgenic Tomato Expressing One or Two Viral Coat Proteins. *Mol. Brrd.,* **5:** 111-119.

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

21. Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P. and Tumer, N. E. 1990. Engineering Resistance To Mixed Virus Infection in a Commercial Potato Cultivars: Resistance to *Potato Virus X* And *Potato Virus Y* In Transgenic Russet Burbank. *Bio. Tech.,* **8:** 127-134.
22. Li, F. and Ding, S. W. 2006. Virus Counterdefense: Diverse Strategies for Evading the RNA-silencing Immunity. *Annu. Rev. Microbiol.,* **60:** 503–531.
23. Lindbo, J., Silva-Rosales, L. and Dougherty, W. 1993a. Pathogen Derived Resistance to Potyviruses: Working but Why? *Semin. Virol.,* **4:** 369-379.
24. Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. and Dougherty, W. G. 1993b. Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resis-tance. *Plant Cell,* **5:** 1749-1759.
25. Morroni, M., Thompson, J. R. and Tepfer, M. 2008. Twenty Years of Transgenic Plants Resistant to *Cucumber Mosaic Virus*. *MPMI,* **21:** 675-684.
26. Murashige, T. and Skoog, F. 1962. A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Plant Physiol.,* **15:** 473-497.
27. Nelson, R. S., Powell-Abel, P. and Beachy, R. N. 1987. Lesions and Virus Accumulation In Inoculated Transgenic Tobacco Plants Expressing the Coat Protein Gene of *Tobacco Mosaic Virus*. *Virol.,* **158:** 126-32.
28. Oyebanji, O. B., Nweke, O., Odebunmi, O., Galadima, N. B., Idris, M. S., Nnodi, U. N., Afolabi, A. S. and Ogbadu, G. H. 2009. Simple, Effective and Economical Explant-Surface Sterilization Protocol for Cowpea, Rice and Sorghum Seeds. *African J. Biotec.,* **8:** 5395-5399.
29. Pares, R. D., Martin, A. B. and Morrison, W. 1985. Virus-induced Tip Necrosis of Passionfruit (*Passiflora edulis* Sims). *Aust. Plant Pathol.,* **14:** 76-78.
30. Peasley, D. and Fitzell, R. D. 1981. Passionfruit Industry Benefits through Scionwood Scheme. *Agric. Gazette NSW,* **92:** 5-8.
31. Powell, A. 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**.** *Sci.,* **232:** 738-743.
32. Rabinowicz, P. D., Bravo-Almonacid, F. F., Lampasona, S., Rodríguez, F., Gracia, O. and Mentaberry, A. N. 1998. Resistance against *Pepper Severe Mosaic Potyvirus* in Transgenic Tobacco Plants. *J. Phytopathol.,* **146:** 315-319.
33. Reichmann, J. L., Latin, S. and Garcia, J. A. 1992. Highlights and Prospects of Potyvirus Molecular Biology. *J. Gen. Virol.*, 73: 1-16.
34. Saris, P. E. J., Paulin, L. G. and Uhlén, M. 1990. Direct Amplification of DNA from Colonies of *Basillus subtilis* and *Escherichia coli* by the Polymerase Chain Reaction. *J. Microbiol. Method.,* **11**: 121-126.
35. Shukla, D. D., McKern, N. M. and Ward, C. W. 1988. Coat Protein of Potyviruses. 5. Symptomology, Serology, and Coat Protein Sequences of Three Strains of Passionfruit Woodiness Virus. *Arch. Virol.,* **102:** 221-232.
36. Smith, H., Swaney, S., Parks, D., Wemsman, E. and Dougherty, W. 1994. Transgenic Plants Virus Resistant Mediated by Untranslatable Sense RNA: Expression, Regulation, and Fate of Nonessential RNAs. *Plant Cell,* **6:** 1441-1453.
37. Smith, H. A., Powers, H., Swaney, S., Brown, C. and Dougherty, W. G. 1995. Transgenic *Potato Virus Y* Resistance in Potato: Evidence Foran RNA-Mediated Cellular Response. *Phytopathol.,* **85:** 864-870.
38. Sokhandan, N., Gillings, M. R. and Bowyer, J. W. 1997. Polymerase Chain Reaction Detection and Assessment of Genetic Variation in New South Wales Isolates of Passionfruit Woodiness Potyvirus. Austral. *Plant Pathol.,* **26:** 155-164.
39. Taylor, R. H. and Kimble, K. A. 1964. Two Unrelated Viruses which Cause Woodiness of Passion Fruit (*Passiflora edulis* Sims). *Aust. J. Agric. Res.,* **15:** 560-570.
40. Vance, V. and Vaucheret, H. 2001. RNA Silencing in Plants-Defense and Counterdefense. *Sci.,* **292:** 2277-2280.
41. Wang, Y. Y., Gardner, R. C. and Pearson, M. N. 1997. Resistance to Vanilla Necrosis Potyvirus in Transgenic Nicotiana Benthamiana Plants Containing the Virus Coat Protein Gene. *J. Phytopathol.*, **145:** 7-15.
42. Worrall, D. 1998. PCR Analysis of Transgenic Tobacco Plants. In: *"Plant*

[DOR: 20.1001.1.16807073.2012.14.5.7.5]

Random Amplified Ploymorphic DNA (RAPD) Analysis in Plants. In: *"PCR Technology, Current Innovations"*, (Eds.): Griffin, H. G. and Griffin, A. M.. CRC Press, Boca Raton, PP. 193-200.

43. Yu, K. and Pauls, K. P. 1994. Optimization of DNA-extraction and PCR Procedures for *Virology Protocols From Virus Isolation to Transgenic Resistance*", (Eds.): Foster, G. D. and Taylor, S. C.. Humana Press, New Jersey, pp571.

یک سازۀ پروتئینی دوگانه، مقاومت به ویروس های چوبی شدن پشنفروت و موزاییک خیار را فراهم می آورد

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چکیده

براساس میزان بالای (>۹۵٪) شباهت داخل گونه ای در ترادف اسید آمینه ای پروتیین کپسیدی ویروس چوبی شدن پسیونفروت (PWV) و ویروس موزاییک خیار (CMV) که هر دو درختچۀ پسیونفروت را در ایالت نیوساث ولز استرالیا آلوده می کنند یک سازۀ دوگانۀ ترانسفورماسیون گیاهی حاوی دی ان ای مکمل کپسید پروتئینی از هر دو ویروس تهیه گردید که در آن ژن کپسید پروتیینی (CP) ویروس موزاییک خیار قبل از PWV CP قرار داده شد. گیاهان نیکوتیانا بنتامیانا که حساس به هر دوی این ویروس ها است از طریق میانجگری آگروبکتریوم تومفسیانس حاوی وکتور دوگانۀ pBI121 که در بردارندۀ سازۀ دوگانه بجای ژن گاس بود ترانسفورم شد. این ترانسفورماسیون با کشت همزمان آگروبکتریوم و تکه برگ های بریده شدۀ گیاه که از برگ های استریلیزۀ سطحی شده تهیه شده بودند انجام گردید. چهار لاین ترانسژنیک شامل ۱-۱، ۱-۵، ۱-۷، ۱-۱۲ و ۱-۲۴ باززایی شدند. درج و نسخه برداری از سازۀ دوگانه مورد تأیید قرار گرفت اما فقط ردیابی CMV CP قابل انجام با الایزا بود. وجود غلظت های پایین تر ویروس در آزمایشات مایه زنی مشهود بود. در آزمایش اولیه که ۱:۱۰ شیرۀ گیاهی مورد استفاده قرار گرفت تأخیر در علایم عموماً مشهود بود. مایه زنی با رقت ۱: ۱۰۰ شیرۀ گیاهی نتایج مشابه با آنچه که از رقت ۱:۱۰ بدست آمده بود تولید کرد. لاین های ۱-۵ و ۱-۱۲ با رقت ۱:۱۰۰۰ شیرۀ گیاهی آزموده شند که تا روز ۲۸ پس از مایه زنی آلودگی با CMV نداشتند اما در قبال PWV لاین ۱-۱۲ تا روز ۱۱ آلوده شده بود. کلون های لاین ۱-۱۲ عکس العمل های متفاوت از خود در برابر مایه زنی ها نشان دادند بطوری که ۳ تا از ۴ لاین در برابر PWV مقاومت نشان دادند در حالی که در قبال CMV دو تا از آنها آلوده نشدند. رابطه ای بین بیان پروتیین و مقاومت محرز نبود و نتایج حاکی از مقاومت مبتنی بر خاموشی آر ان ای بود.

[DOR: 20.1001.1.16807073.2012.14.5.7.5]