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Full Length Research Paper

Testing of disease-resistance of pokeweed antiviral protein gene (*PacPAP*) in transgenic cucumber (*Cucumis sativus*)

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Transformation of pokeweed antiviral protein gene (*PAP*) into plants was shown to improve plant resistance to several viruses or fungi pathogens with no much negative effect on plant growth. The non-virulent defective PAP inhibits only the virus but does not interfere with the host. A non-virulent defective PAP gene (*PacPAP*) from *Phytolacca acinosa* was introduced into cucumber successfully by agrobacterium mediated method. Southern blotting and northern blotting analyses indicated that, 5 stable transgenic cucumber lines with *PacPAP* were obtained and *PacPAP* showed different expression levels in transgenic plants. The identification of resistances to disease was performed by artificial inoculation of cucumber mosaic virus (CMV) and *Fusarium oxysporum* fsp*. cucumerinum* on T_0 and T_1 transgenic plants. Compared with the non-transgenic susceptible plants, all transgenic plants with *PacPAP* showed resistance in different degree to CMV and the CMV-resistance of progeny (T_1) from transgenic lines could inherit stably, but the transgenic plants did not resist to Fusarium wilt of cucumber, the *PacPAP* was not resistant to *Fusarium oxysporum* fsp*. cucumerinum*. This work provides a new virus resistant cucumber breeding resource.

Key Words: Pokeweed antiviral protein gene, genetic transformation, *Cucumis sativus*

INTRODUCTION

Viral diseases, caused by tobacco mosaic virus, potato virus X and Y, cucumber mosaic virus, tunip mosaic virus and cauliflower mosaic virus, play a major role in endangering vegetable crops of Solanaceae and Cruciferae families. Compared with diseases caused by other pathogens, viral diseases are more difficult to be cured by conventional chemical methods and so far an efficient chemical method is still unavailable. Virus resistance, previously observed in transgenic plants expressing coat protein genes, has been specific for the virus from which the genes are derived or closely related viruses (Lee et al., 2009). The results of the experiments described here provide a way of producing transgenic plants that may be resistant to a broad spectrum of plant viruses.

Pokeweed antiviral protein (PAP) is an alkaline protein from plants of *Phytolacca* genus, it belongs to a group of ribosome-inactivating protein I (RIP I), which inhibits the effect of plant virus, fungi and bacteria (Irivin, 1975; Irivin et al., 1980; Irivin and Uckun, 1992; Lin et al., 1991; Kataoka et al., 1992; Kung et al., 1990, Hudak et al., 2004). The purified PAP from *Phytolacca americana* leaves is shown to inhibit seven different viruses (Chen et

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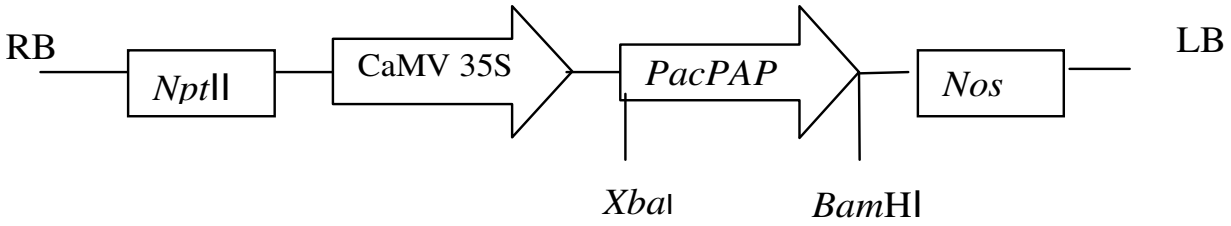

Figure 1. The pattern of expression vector pBI-*PacPAP*.

al., 1991). The purified protein can also inhibit the replication of flu virus and even HIV in vivo (Tomlinson and Walke, 1974; Zarling et al., 1990). The transgenic plants with PAP genes have larger antiviral spectra. Monsanto Company has successfully introduced PAP gene into potato, which enables transgenic plants to acquire large spectra antiviral capacity, including the virus propagated by physical wounding and aphids. PAP-transgenic plants, such as tobacco (Smironv et al., 1997; Wang et al., 2008), pepper (Chen et al., 2008), citrus (Petersen et al., 2003), mustard (Zhao et al., 2008), potato (Fu et al., 2000) and *Brassica napus* (Zhang and Dang, 1999), all acquired significant virus-resistance. Furthermore, PAP-transgenic plants also acquired resistance to fungi. Wang et al. (1998) obtained PAP II transgenic tobacco plants with resistance to fungi *Rhizoctonia solani* and also, resistances to tobacco mosaic virus U1 (TMVU1) and potato virus X (PVX). Based on its characteristic cell toxicity, the PAP and its specific antibody were used to produce immunotoxin against human viral diseases, cancer and tumours (Aron and Irvin, 1980; Olson et al., 1991; Zarling et al., 1990). However, transgenic tobacco or potato plants with PAP gene displayed reduced growth (Nilgun et al., 1998). Transgenic tobacco with a deletion of partial C terminal PAP had normal growth and virus resistance, including aphis and physical wounding mediated cucumber mosaic virus (CMV), PVX and potato virus (PVY) (Lodge et al., 1993). Viruses are some of the most important pathogens of cucurbits. Diseased plants typically show leaf mosaic, puckering and stunting symptoms and their misshapen fruits are often un-marketable (Pink and Walkey, 1985a). About 20 different viruses are known to cause economic losses on cucurbits. Throughout the temperate regions, CMV is one of the most common viruses, infecting 775 plant species belonging to 85 families including Cucurbitaceae (cucumbers, melons, squashes, pumpkins) (Lovisolo, 1980). If infection occurs at the seedling stage, the disease may completely destroy the crop. Fusarium wilt of cucumber, caused by soil-borne necrotrophic fungus *Fusarium oxysporum* fsp.*cucumerinum*, is also a serious vascular disease worldwide. The disease is a yield-limiting factor in cucumber production and it occurs at all growing stages of cucumber (Ye et al., 2004). Few effective, economical and environmental safe management practices are available in the control of this disease and highly resistant cultivars have not been developed (Vakalounakis et al., 2004).

The lack of disease resistance resources affects the cucumber breeding. Previous studies showed that, the toxic region to virus and toxic region to host plants are separate in the PAP gene and therefore, it is possible to keep the viral resistance region without affecting the host plants via gene modification, which could improve its range of application (Tumer et al., 1997). A toxic-PAP gene (*PacPAP*) was cloned from *Phytolacca acinosa* and introduced into the pepper, the transgenic pepper showed high resistance to TMV and CMV (Chen et al 2008). This study was aimed to introduce *PacPAP* into cucumber to provide a new resistant to disease resource for cucumber breeding.

MATERIALS AND METHODS

Plant, *Agrobacterium tumefaciens* strain and plasmid

The cucumber inbred line ('02-8', susceptible CMV) (*Cucumis sativus* L.) was from our laboratory. CMV inoculation was performed using CMV infected cucumber seedling leaves; CMV was isolated from the CMV-infected cucumber plant growing in the field of South China Agricultural University (SCAU).

The expression vector pBI-*PacPAP* (Figure 1) with CaMV35S promoter and *npt*II gene was constructed and transferred into *A. tumefaciens* strain EHA105 by freeze-thaw method (Chen et al., 2008).

Agrobacterium-mediated transformation into cucumber

Agrobacterium mediated transformation of cotyledons obtained from sterile 8 days old cucumber seedlings were performed. Briefly, the seedlings were cut off and the tips were removed and sectioned transversely with a scalpel into two fragments. Cotyledon pieces were placed upside down in 90×15 mm Petri dishes containing pre-culture medium MS (Murashige and Skoog, 1962) + (6-BA 1 .0 mg L^{-1} + $AgNO_3$ 2.0 mg L^{-1} + sucrose 30 g.L^{-1} + agar 6.5 g.L^{-1}, pH5.8) and incubated for 2 days at 25°C in the dark. Cotyledon explants were then removed from the pre-culture plates and transferred to the Agrobacterium suspension for 20 min with gentle shake for several times (all cotyledon explants should be immersed). The explants were blotted dry using filter paper and then, were placed on co-culture medium (MS + 6-BA1.0 mg L^{-1} + $AgNO_3$ 2.0 mg L^{-1} + sucrose 30 g.L^{-1} + agar 6.5 g.L^{-1}, pH5.8) for 2 days at 25°C in the dark. Cotyledon explants were then further transferred to selective

medium (MS + 6-BA1.0 mg L^{-1} + $AgNO_3$ 2.0 mg L^{-1} + Kanamycin 70 mg L^{-1} + cefotaxime 300 mg L^{-1}+ sucrose 30 g.L^{-1} + agar 6.5 g.L^{-1}, pH5.8). Four weeks later, when regenerated plantlets reached 2 to 3 cm in height, the Km-resistant buds were cut off and were cultured on differentiation medium (MS + 6-BA1.0 mg.L^{-1} + $AgNO_3$2.0 mg L^{-1}+ sucrose 30 g.L^{-1} + agar 6.5 g.L^{-1}, pH5.8) for 2 to 3 subculture and placed on a rooting medium (MS + Kanamycin 70 mg L^{-1} + cefotaxime 200 mg.L^{-1}) in sterile plant containers. After 20 days, plants that developed roots were transplanted into greenhouse for further analysis.

DNA and RNA extraction

Total genomic DNA was extracted by CTAB methods (Doyle et al., 1990). Total RNA was extracted by RNA extraction Trizol kit (Takara.com).

Polymerase chain reaction amplification (PCR) and RT-PCR analysis

Genomic DNA was extracted from 3 to 5 g fresh weight leaf according to CTAB method. Conventional PCR was used to amply *PacPAP* genes to check the insertion of target gene fragment. The primers were designed according to *PacPAP* (AY603353) sequence. The forward and reverse primer sequences were *PAP*-F:5'-CCTCTAGA CATATGGTGAATACAATCATCTACAA-3', *PAP*-R :5'-AAGGTACC GGATCCTCACCACTTGGCACCACTGG -3'. The PCR reactions were carried out in a 25 µl volume containing 2.5 µl 10×PCR buffer (TaKaRa), 0.5 µl 10 mmol.l^{-1} dNTPs (TaKaRa), 0.5µl 20 µM primer, 0.5 µl 5U/µl *Taq* polymerase (TaKaRa), 20 µl distilled H_2O and 1 µl (20 to 50 ng) DNA template. Reactions were carried out in a peltier thermal cycler (Bio-Rad, USA) as follows: one cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 90 s at 50°C, 30 s at 72°C and one final cycle of 10 min at 72°C. The products were separated in 1.2% agarose with electrophoresis systems (Bio-Rad, sub-cell model 192, USA) and bands were recorded using a Chemi Doc system (Bio-Rad, USA).

Trangenic plant total RNA was extracted by Trizol method. Reverse transcription and sub- sequential PCR was performed according to protocol of TaKaRa RNA PCR Kit (AMV) Ver. 3.0.

Southern blotting analysis

Approximately, 10 µg DNA from transformed and control plants were digested with *BamH* I for 12 h at 37°C overnight and separated by electrophoresis in a 0.8% agarose gel. DNA was transferred to a nylon membrane and hybridized with a radioactive *PacPAP* gene fragment probe. Hybridization was carried out by DNA labelling and detection kit (Boehringer Mannheim Com.). The membrane was washed at high stringency in which the solution contains 0.2×SSC, 0.1% SDS at 65°C and then, detected using BIO-RAD FX system.

Northern blotting analysis

Hybridization was carried out by DNA labelling and detection kit (Boehringer Mannheim Com.). Approximately, 12 µg of the total RNA was run on a 1.2% denatured agarose gel containing formaldehyde and then, transferred onto positively charged nylon membrane (Boehringer Mannheim com). The DIG-labelled *PacPAP* gene fragment was used as the probe.

Antiviral activity detection on transgenic plants

The CMV (yellow strain) was originally isolated from *C. sativus* L.

About 1 g fresh cucumber leaves with CMV symptoms was ground in a mortar and 10 ml of phosphate buffered saline with pH 7.4 was added. The extract was filtered using gauze and the filtrate was inoculated on the cucumber plants by friction. Cucumber seedlings with 3 to 4 leaves were used for inoculation. Second inoculation was performed followed by the third inoculation to ensure the infection. Plants were grown in an insect-free room at 25 to 28°C or 20°C day/night temperature. The disease index (DI) was evaluated after 25 days inoculation. Disease severity was assessed on a 0 to 9 scale: Level 0, no symptom; level 1, one or two mosaic leaves; level 3, all upper half of plant contained mosaic leave chlorotics; level 5, most leaves contained mosaic and abnormal leaves, leaf deformity; level 7, all mosaic leaves, dwarf plants; level 9, all mosaic leaves, plant growth severely inhibited or plant wilt and death. 0 to 3 for resistance and 5 to 9 for susceptibility.

CMV-antiviral population classification: High resistance (HR), DI 0-5; resistant (R), DI 5-20, moderately resistant (MR), DI 20-40; susceptible (S), DI above 40.

Testing *Fusarium oxysporum* fsp.*cucumerinum* on transgenic plants

F. oxysporum fsp. *cucumerinum* was extracted from susceptible disease cucumber plant. Culture of the pathogen isolation was grown in potato dextrose broth (PDB) in 200 ml Erlenmeyer flasks in a rotary shaker for 6 days at 22°C in the dark. Spores were removed from the substrate, filtered through a double layer of non-sterile cheesecloth and the mycelial mat was washed with distilled water and the suspension centrifuged at 3000 g for 10 min. Spores were resuspended in distilled water and the concentration was adjusted to 1 × 10^6spores/ml.

At the two or three leaves stage, the seedlings were inoculated. The plants were removed from the substrate and washed with tap water before inoculation and then, dipped in the inoculum suspension for 3 to 5 min. After inoculation, seedlings were transplanted in sterile organic substrate. After being inoculated for 15 days, the disease symptom was investigated. To evaluate the response of seedlings to *F. oxysporum* fsp. *cucumerinum, an* arbitrary five-point visual scale was used; where, 0 = no apparent symptoms; 1 = area of yellow or wilting leaf <50%; 2= area of yellow or wilting leaf more than 50%; 3= area of wilting or dead leaf more than 50%; 4 = the whole plant dead. When calculating segregation ratios, only plants with a disease rating of 0 and 1 were classed as resistant, while those with disease ratings of 2, 3 and 4 were classed as susceptible.

Scale for evaluation of resistance to disease of plants by disease index (DI): High resistant (HR) DI 0 < 10, resistant (R) 10 ≤ DI < 3, moderately resistant (MR) 30 ≤ DI < 50, susceptible (S) 50 ≤ DI < 70 and highly susceptible (HS) DI ≥ 70, respectively.

RESULTS

Regeneration and detection of transgenic plants

About 2000 cotyledons were selected, most of the explants failed to differentiate and turned yellow, but some Kan^r adventitious bud rosettes were differentiated from the cut end of cotyledons explants after 25 days culturing on selective medium. These buds were selected by more times. Only 5 Kan-resistant buds were obtained at last and the 5 transgenic T_0 plants (C_5, C_{11}, C_{33}, C_{63}, C_{90}) were analyzed by southern blotting (Figure 2). DNA samples of kan-resistant buds and non-transformed (as control) cucumber were isolated from leaves. Southern

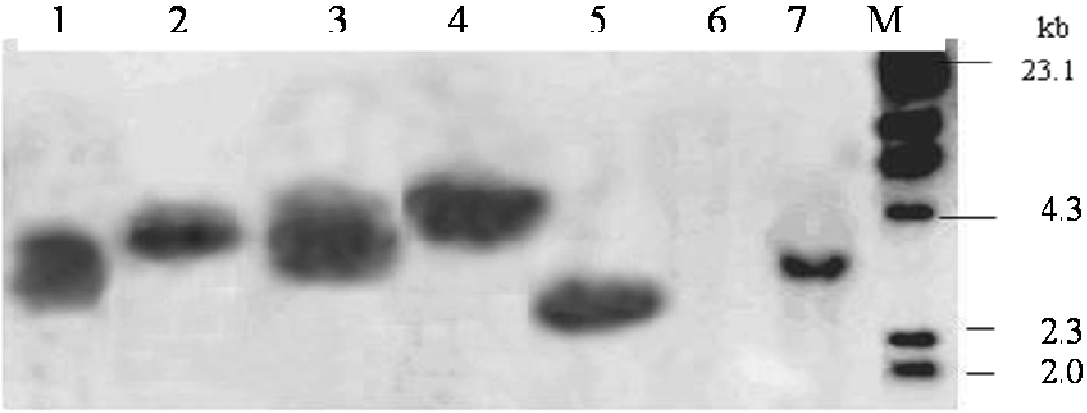

Figure 2. Analysis of Southern blotting of transgenic plants. M, Marker; lanes 1 to 5, transgenic lines (C_5,C_{11},C_{33},C_{63},C_{90}); lane 6, non-transgenic plant; lane 7, positive CK (plasmid pBI-*PacPAP*).

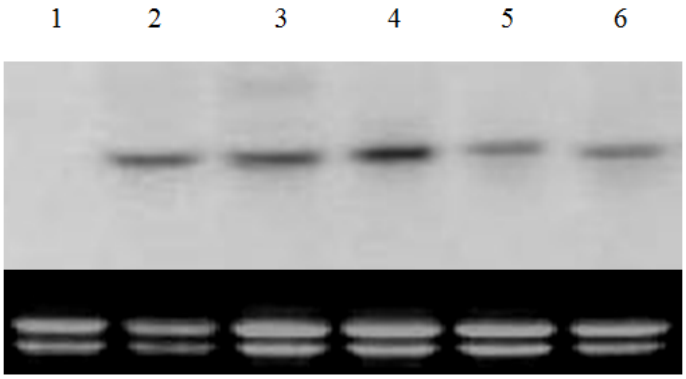

Figure 3. Northern blotting analysis of transgenic plants. Lane 1, the *PacPAP* gene expression in the leaf of the non-transgenic plant; lanes 2 to 6, the *PacPAP* gene expression in the leaves of the T_0 transgenic lines (C_5,C_{11},C_{33},C_{63},C_{90}).

hybridization demonstrated that, *PacPAP* gene had been integrated into the genome of the transgenic lines and was all single copy. These transgenic plants were propagated. After the roots of the shoots were induced on the rooting medium, they were transplanted into vermiculite.

After extraction of the total RNA from the transformation plants leaves, the expression of *PacPAP* gene was analyzed by northern blotting. The results showed that, the target genes were expressed in transgenic plants, but not in non-transgenic plants and the expression level was different in various transgenic plants (Figure 3).

Detection of antiviral activity of transgenic plants (T_0)

Compared with the wild-type plants, the transgenic cucumber plants did not show any observable change in their growth performance and they had similar botanical and biological characteristics to the wild-type (Table 1). Similar to the wild-type counterpart, the transgenic plants were self-fertile and set fruit by self-pollination.

The transgenic T_0 plants and the wild-type plant were tested by inoculating CMV in greenhouse and field. The inoculation of CMV was preformed to detect the antiviral activity of transgenic T_0 plants. About 20 days after inoculation, the wild-type plants presented typical CMV symptom such as present mosaic leaves, while very few symptoms appeared in the transgenic plants (Figures 4a, b). 25 days after inoculation, the CMV resistances of these plants were evaluated in greenhouse (Table 2). Under the same conditions, the transgenic and control plants were transferred to the field, the wild-type plants developed serious symptoms of the viral infection and some of the control plants eventually died from the CMV.

Table 1. Comparison the characterization of wild-type plant with that of the transgenic plants (T_0).

Material	Plant height (m)	Fruit Length (cm)	Fruit stalk length (cm)	Fruit length /stalk ratio	Fruit Diameter (cm)	Per fruit weight (g)	First flower node	First female flower node
CK	2.24± 0.12	32.3±2.0	3.8±0.2	8.56±0.21	3.56±0.13	235.3±3.3	3.1±0.1	5.2±0.2
C_5	2.19±0.11	31.5±1.9	3.7±0.1	8.57±0.23	3.53±0.12	231.4±4.1	3.1±0.2	5.2±0.1
C_{11}	2.19±0.10	32.7±2.3	3.8±0.2	8.63±0.31	3.61±0.24	235.6±3.0	3.2±0.1	5.1±0.3
C_{33}	2.25±0.16	30.8±1.8	3.6±0.1	8.65±0.21	3.58±0.16	230.7±4.5	3.2±0.1	5.2±0.1
C_{63}	2.20±0.15	31.3±1.9	3.7±0.3	8.44±0.26	3.51±0.27	234.3±3.5	3.2±0,2	5.3±0.1
C_{90}	2.18±0.13	31.7±1.8	3.8±0.3	8.37±0.22	3.60±0.31	234.8±3.6	3.1±0.2	5.3±0.2

The height of plants with 20 leaves was tested.

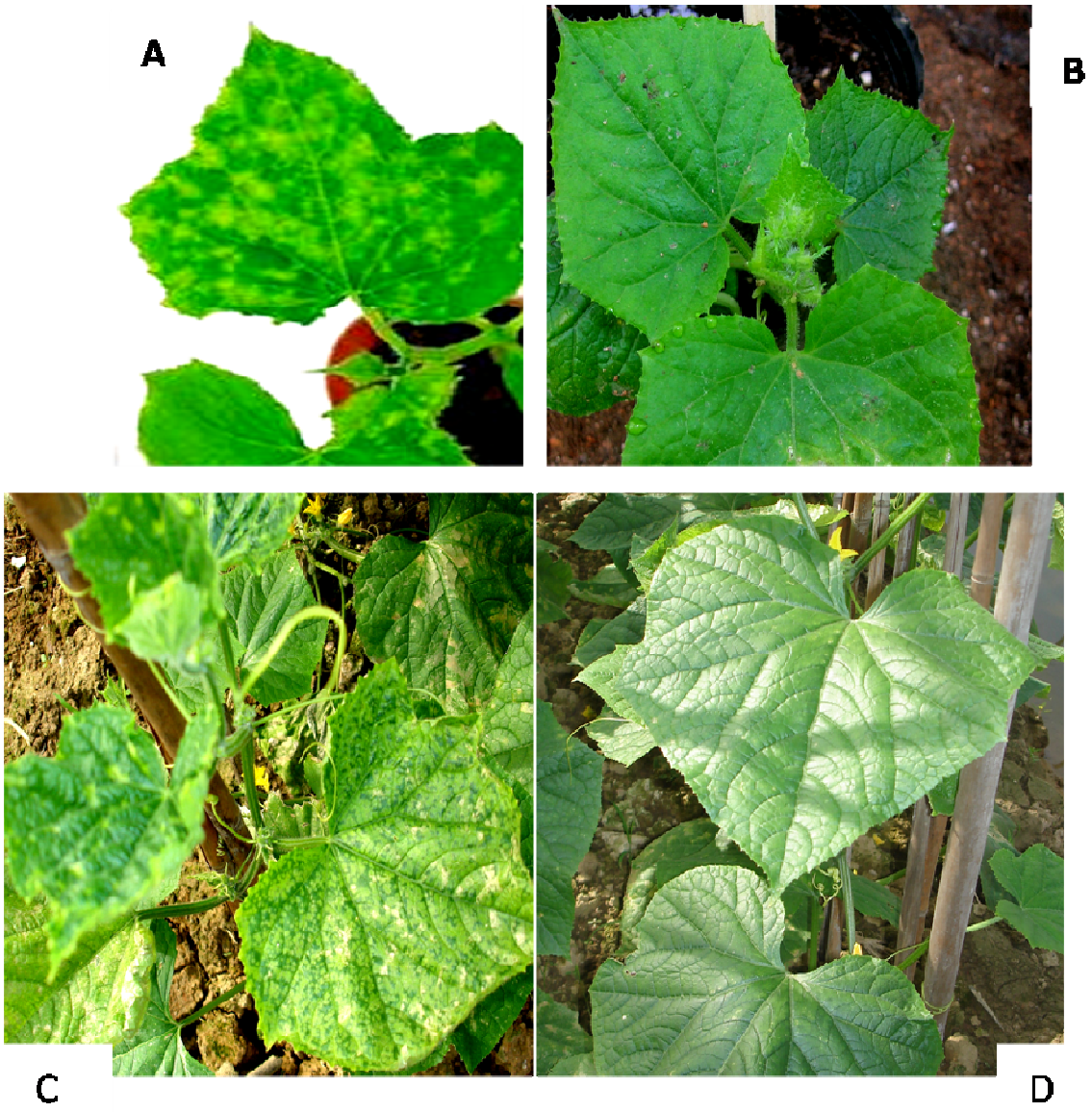

Figure 4. Testing CMV-resistance of transgenic plant in the greenhouse and the field. A, Inoculation CMV on non-transgenic plant (CK); B, inoculation CMV on the transgenic line (C_{90}); C, CMV infection of non-transgenic plant in the field (CK).

Table 2. Testing results of CMV inoculation of transgenic lines (T_0).

T_0 transgenic line	Number of plant	Disease scale						DI	Resistance of population
		0	1	3	5	7	9		
CK	95	0	0	0	5	30	60	90.6	Susceptible
C_5	61	13	30	18	0	0	0	14.1	Resistant
C_{11}	66	8	37	21	0	0	0	16.8	Resistant
C_{33}	62	16	28	18	0	0	0	14.9	Resistant
C_{63}	62	12	36	14	0	0	0	14.0	Resistant
C_{90}	60	11	37	12	0	0	0	13.5	Resistant

DI, Disease index.

Table 3. Testing results of *F. oxysporum* fsp.*cucumerinum* inoculation of transgenic lines (T_0).

T_0 transgenic line	Number of plant	Disease scale					DI	Resistance of population
		0	1	2	3	4		
CK	67	0	4	18	21	28	80.2	Susceptible
C_5	76	1	10	16	25	25	71.4	Susceptible
C_{11}	65	0	11	12	18	24	71.2	Susceptible
C_{33}	65	0	3	12	20	30	79.6	Susceptible
C_{63}	70	2	6	15	23	24	71.8	Susceptible
C_{90}	66	0	7	11	26	22	73.9	Susceptible

80% plants were affected by CMV disease, but the transgenic T_0 plants did not display any symptoms of CMV infection during this period of field growth (Figures 4 c, d). The results showed that the CMV-resistance of all transgenic plants was increased.

Testing of *F. oxysporum* fsp.*cucumerinum* in transgenic plants (T_0)

In order to test the resistance to *F. oxysporum* fsp.*cucumerinum* in transgenic T_0 plants, five transgenic lines (C_5, C_{11}, C_{33}, C_{63}, C_{90}) were propagated, 90 plants of each and wild-type plants were inoculated with *F. oxysporum* fsp.*cucumerinum* and the results indicated that, DI of all transgenic plant and wild-type plant were more than 70 (Table 3), they showed high susceptible to disease and the *PacPAP* gene had not the function of resistance to *F. oxysporum* fsp.*cucumerinum*.

Analysis of the CMV-resistance of the T_1 progenies of self pollinated transgenic lines

These transgenic T_0 plants were self-pollinated and the T_1 generation seeds ($C_{5\text{-}1}$, $C_{11\text{-}1}$, $C_{33\text{-}1}$, C_{63}, $C_{90\text{-}1}$) were obtained and sowed in greenhouse to grow T_1 plants. PCR analysis using *PacPAP* primers was preformed on the T_1 progenies plants from all five transgenic lines to detect the presence to insertion of target genes. 120 T_1 plants of each transgenic line were identified (Figure 5) and the results showed the separation ratio of progenies plants having target gene and no target gene from all transgenic lines showed 3:1. The CMV-resistance of progenies plants from all five transgenic lines were evaluated, the result showed that in all cases, the presence of *PacPAP* gene co-segregated with the CMV-resistance trait (Table 4) and the *PacPAP* gene could inherit stably in the progenies of transgenic plants.

DISCUSSION

Up till now, there are many reports on the transformation of PAP gene to increase the disease-resistance of plants, but the defense mechanisms of PAP were argued, Zoubenko et al. (1997) thought PAP might directly attack a pathogen by affecting viral nucleic acid or by depurinating fungal ribosomes. Alternatively, the expression of PAP might activate host defense pathways and lead to broad-spectrum resistance to pathogen infection, similar to systemic acquired resistance (SAR), which is characterized by activation of a signal transduction pathway and synthesis of a number of defense gene products. Lodage et al. (1993) thought the transformation of PAP gene could affect the growth of transgenic plants, such as transgenic tobacco and potato plant with full length PAP (313 amino acids) displayed reduced growth. However, in this experiment the transgenic cucumber plants (T_0) did not show any observable change in their growth

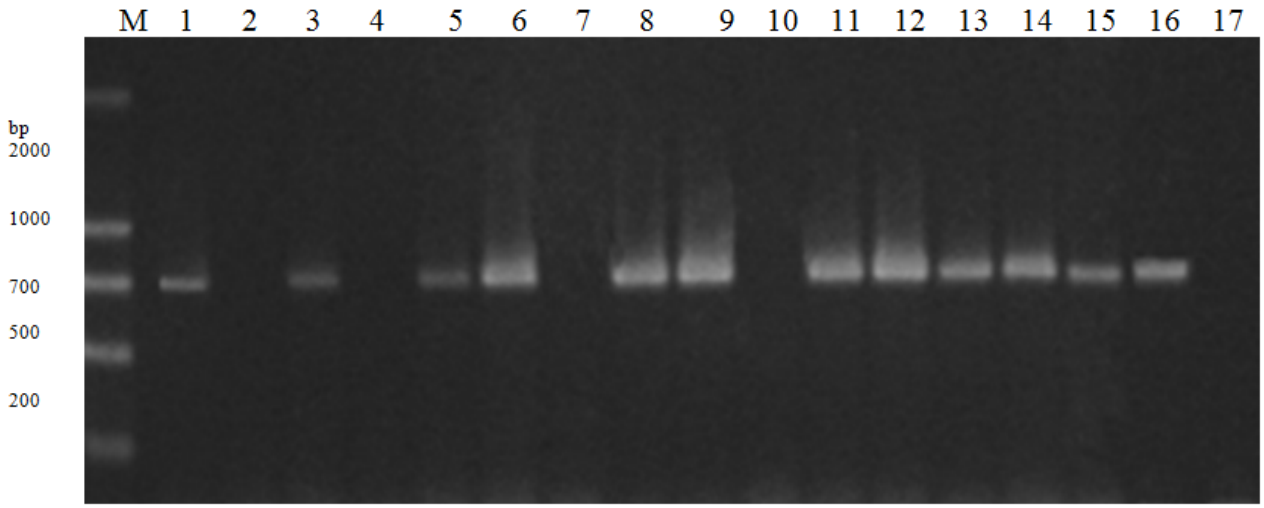

Figure 5. The PCR detection *PacPAP* of T_1 progenies of transgenic plant (C_{90-1}). M, Marker; lane 1, positive control (pBI-*PacPAP)*; lane 2, non transgenic plant; lanes 3 to17, T_1 progenies plants of transgenic line C_{90-1}.

Table 4. Testing CMV-resistance of T_1 progeny plants from transgenic lines.

T_1 progenies of transgenic line	Number of total plants	Number of CMV-resistance plant	Number of CMV-susceptible plant	Ratio(R/S)	$\chi^2_{0.05}$=3.84
C_{5-1}	120	93	27	3:1	0.28
C_{11-1}	120	86	34	3:1	0.54
C_{33-1}	120	92	28	3:1	0.10
C_{63-1}	120	88	32	3:1	0.10
C_{90-1}	120	89	31	3:1	0.01

performance compared with the wild-type and they had similar botanical and biological characteristics to wild-type, it may be due to non-virulent defective fragment of PAP (Chen et al, 2008). The ORF of PAP genes comprise 942 bp and encode for a protein of 313 amino acids (Lin et al., 1991). Previous studies indicated that, there are different functional domains in PAP gene. It was demonstrated that, the domain of 170th to 187th amino acid in N terminal is a highly conserved active functional domain, which confers antiviral activity; the domain of 1st to 25th amino acid in the C terminal has the activity to cut off an adenine in special site of rRNA, which was showed to be toxic to host plants and was independent to antiviral activity (Lin et al., 1991; Tumer et al., 1997). According to 3D analysis of PAP protein structure, a minimum of 63 amino acids are required to form a mature protein structure and this structure could separate the antiviral activity domain and host plant-toxic domain (Tumer et al., 1997). It was also showed that, C-terminal 1st to 25th amino acids missing PAP was not toxic to host plant cell but still kept its antiviral activity and a functional protein (Tumer et al., 1997). However, mutation in highly conserved antiviral domain for example institute E to V in 176th amino acid, will lead to loss protein antiviral activity (Tumer et al., 1997). In this study, we first introduced a toxic-free PAP gene (*PacPAP*) to CMV susceptible cucumber and obtained transgenic plants with normal growth and antiviral properties. This indicated that, the antiviral domain could be functional without its host cell toxic domain. Our results also indicated that, it was possible to use PAP gene via genetic engineering with a functional antiviral activity without any toxicity to the host cell. Some reports showed that, the transgenic PAP gene plants had fungi pathogen-resistance, but in this study, all transgenic plant was not resisted to *F. oxysporum* fsp.*cucumerinum*, PAP gene could not resist to broad spectrum fungi pathogens in plant.

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