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ORIGINAL RESEARCH PAPER

# Expression of a polyphemusin variant in transgenic tobacco confers resistance against plant pathogenic bacteria, fungi and a virus

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**Abstract** Antimicrobial cationic peptides provide a promising means of engineering plant resistance to a range of plant pathogens, including viruses. PV5 is a synthetic structural variant of polyphemusin, a cationic peptide derived from the horseshoe crab-*Limulus polyphemus*. PV5 has been shown to be benign toward eukaryotic membranes but with enhanced antimicrobial activity against animal pathogens. In this work, the cytotoxicity of PV5 toward tobacco protoplasts and leaf discs was assessed using TTC (2,3,5-triphenyltetrazolium chloride) and Evans blue colorimetric assays. PV5 showed no measurable cytotoxic effects even at levels as high as 60 $\mu$g. As a possible approach to enhancing plant resistance, a gene encoding PV5 was fused to the signal sequence encoding the C-terminus portion of the BiP protein from *Pseudotsuga menziesii*, under the control of $2 \times 35$S CaMV promoter. When introduced into *Nicotiana tabacum* var Xanthi gene integration and expression was confirmed by both Southern and northern analyses. When transgenic plants were subsequently challenged with bacterial and fungal phytopathogens enhanced resistance was observed. Moreover, transgenic plants also displayed antiviral properties against Tobacco Mosaic Virus making PV5 an excellent candidate for conferring unusually broad spectrum resistance to plants and the first anti-plant virus antimicrobial peptide.

**Keywords** Cationic peptides · Protoplast · Transgenic · Cytotoxicity

**Abbreviations**

| | |
|---|---|
| CAP | Cationic antimicrobial peptides |
| $dH_2O$ | Distilled water |
| HIV | Human immunodeficiency virus |
| MS | Murashige–Skoog |
| TMV | Tobacco mosaic virus |
| TTC | 2,3,5-Triphenyltetrazolium chloride |

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## Introduction

Cationic antimicrobial peptides (CAPs) play a critical role in innate defense of most living organisms against microbial infections (Boman 1995; Hancock and Lehrer 1998). Synthetic

derivatives of these cationic peptides have shown to be more effective against the pathogens and safe toward living cells (Johnstone et al. 2000; Osusky et al. 2000; Pacor et al. 2002). The PV5 is a synthetic derivative of polyphemusin, a CAP derived from horseshoe crab-*Limulus polyphemus*. Polyphemusin and its isopeptide tachyplesin form antiparallel $\beta$-sheet structures stabilized by disulphide bridges. These CAPs have been shown to be active in vitro against bacteria, fungi and even viruses, including vesicular stomatitis virus, influenza A virus, and human immunodeficiency virus (HIV-1) (Allefs et al. 1996; Miyata et al. 1989; Murakami et al. 1991; Nakashima et al. 1992). Polyphemusin analogs were developed by rational design and their mechanism of action previously demonstrated. Three structural variants (PV5, PV7, and PV8) of polyphemusin I were designed with improved amphipathic profiles, decreased activity on eukaryotic membranes and improved activity against bacteria (Zhang et al. 2000). However, there is no report of their cytotoxicity assessment on plants and their activity on plant pathogens.

For expressing heterologous peptides in plants, a thorough evaluation of toxicity not only to humans and animals, but plants is necessary (Franck-Oberaspach 1997). Cytotoxicity of known and characterized cationic peptides, have been tested against animal cells but methods needed to be developed to test their cytotoxicity in plants; cell death being an effective indicator of cytotoxicity. Colorimetric assays for plants have been designed to measure cell viability under stress and in response to chemical treatment (Able et al. 1998; Baker and Mock 1994; Borenfreund and Puerner 1985; Capasso et al. 2003; Mosmann 1983; Steponkus and Lanphear 1967; Towill and Mazur 1974). Colorless, tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 2,3,5-triphenyltetrazolium chloride (TTC), are widely used to assess viability of mammalian and plant cells (Badini et al. 1994; Caldwell 1993; Chang et al. 1999; Parker 1953; Towill and Mazur 1974). Reduction of TTC measures the overall oxidative metabolic processes of the cell. Evans blue is an impermeant dye that leaks though ruptured membranes, and stains the contents of dead cells. It has been used to determine cell death via microscopy (Smith et al. 1982; Taylor 1980) as well as in leaf discs (Baker and Mock 1994).

In the past few years it has become apparent that plants expressing cationic peptides exhibit broad-spectrum resistance including the ability to kill bacteria, fungi and protozoa (Allefs et al. 1996; Florack et al. 1994; Florack et al. 1995; Hancock and Diamond 2000; Osusky et al. 2000; Osusky et al. 2004; Osusky et al. 2005; Ponti et al. 2003; Yevtushenko et al. 2005). Transgenic plants are an important tool to manage a broad-spectrum of diseases and to reduce use of pesticides. Expression of tachyplesin along with a signal sequence in plants has been shown to confer resistance against *Erwinia* soft rot (Allefs et al. 1996). Synthetic cationic peptides (e.g. modified variants of cecropin, melittin, temporin etc.) expression in transgenic potato and tobacco have provided enhanced resistance against a number of plant fungal pathogens, including *Colletotrichum*, *Fusarium*, *Verticillium* and *Phytophthora* sp. (Cavallarin et al. 1998; Osusky et al. 2000; Osusky et al. 2004; Osusky et al. 2005; Yevtushenko et al. 2005). So far there are no reports of expression of cationic peptides in plants and generation of resistance to viruses.

The purpose of this work was to establish effective in vitro assays for plant cell viability and then examine the in vitro antimicrobial activities of PV5 including against several bacterial and fungal phytopathogens as well as a plant virus. TTC and Evan's blue proved to be effective reporters of plant cell cytototoxicity and were successfully used to establish that PV5 was relatively benign toward plant cells. We also report that transgenic expression of PV5 in *Nicotiana tabacum* conferred enhanced resistance to a variety of phytopathogens: bacteria, fungi and TMV. These results highlight the biotechnological potential of polyphemusin variants in plants.

## Materials and methods

### Peptide and chemicals

The CAP used in this study was the synthetic polyphemusin derivative, PV5 (MRRYCYRK-

CYKGYCYRKCR). PV5 was a gift from Dr. R.E.W Hancock, University of British Columbia, Vancouver, Canada. Lyophilized peptide powder (>90% purity) were reconstituted in sterile, deionized water, filtered though a 0.2 $\mu$m Millipore filter and stored at –20°C until further use. Carbenicillin disodium salt was purchased from Invitrogen (Cat. No. 10177–012). Round-up® (Glyphosate isopropylamine) is a commercial herbicide from Monsanto Canada Inc.

## Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) were grown aseptically in Magenta vessels (Magenta, USA) on MS medium containing 2% sucrose (pH 5.8), in a 16 h light photoperiod (25 $\mu$mol $m^{-2}$ $s^{-1}$) at 24°C. Non-transgenic and transgenic plants were transferred to the greenhouse, where they were planted in pots with moistened Sunshine mix # 2 soil (Sun Gro Horticulture, Bellevue, Washington, USA).

## Pathogen cultures

The bacterial strain used in this study was the laboratory isolate of *Erwinia carotovora.* Fungal pathogens used were *Botrytis cineria, Verticillium* sp. and *Fusarium oxysporum* obtained from Dr. Zamir Punja, Simon Fraser University, Burnaby. The plant virus used was Tobacco mosaic virus (TMV) (ATCC 135 PV) obtained from American Type Culture Collection, Atlanta, USA.

## Vector construction

A vector designed to express gene under the control of a 2 × 35S constitutive promoter was used for tobacco transformation. The construct contained the duplicated-enhancer CaMV 35S promoter (2 × 35S) with the AMV RNA4 leader translational activator for high-level production of PV5 gene. The duplicated-enhancer 35S promoter (Kay et al. 1987), exhibits ten times the transcriptional activating properties of the wild type 35S promoter, while the AMV translational enhancer increases translational efficiency by up to four times (Datla et al. 1993). Plasmid, pPV5 contained the PV5 chimeric gene, amplified by gene-specific, overlapping synthetic oligonucleotides. Primers designed for construction of PV5 gene were based on the nucleotide sequences of the peptides, determined by reverse-translation of the amino acid sequences using plant specific codons and modifying them by adding specific restriction enzyme sites for DNA cloning and expression. The synthetic gene for PV5 was assembled by PCR using the oligonucleotides shown in Fig. 3A. It was first cloned into the vector pBI 524; the promoter and PV5 gene were subsequently cloned into pBI 121 for transformation into *Agrobacterium tumefaciens*, The synthetic gene encoding PV5 had *NcoI* and *BamHI* sites at 5′and 3′ ends respectively and this was first cloned in vector pBI 524. A fragment from this vector containing the 2 × 35S promoter with an AMV RNA4 translation-enhancing element (Datla et al. 1993) and PV5 with NOS terminator (NOS-ter) was ligated in place of the deleted GUS-containing region of pBI 121. The T-DNA region of the vector also contained the *NOS*-driven *NPTII* gene, used for the selection of kanamycin-resistant plants (Fig. 3B). The construct was introduced into *A. tumefaciens* MP90 by the freeze–thaw method (Holsters et al. 1978).

## Plant transformation and selection of transgenics

A *A. tumefaciens* MP90 culture harboring the above vectors was grown for 1–2 days at 28°C on a rotary shaker at 225 rpm in liquid LB medium (Sambrook et al. 1989) supplemented with 100 mg $l^{-1}$ kanamycin, 100 mg $l^{-1}$ rifampicin and 10 mg $l^{-1}$ gentamicin. Transformation of tobacco plants was carried out as described by Horsch et al. (1988).

## PCR analysis of transgenic plants

Plant DNA was isolated from tobacco leaves using a GenElute™ Plant Genomic DNA Kit (Sigma, USA). PCR was carried out in 50 $\mu$l reaction mixture containing 200 ng of plant DNA, Taq PCR Master Mix (Qiagen, USA) and specific primers, with manual hot start and the following parameters: 94°C for 3 min, then 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s, followed

by a final 10 min incubation at 72°C. Primers for plants with the pPV5 construct amplified the 110 bp full-length PV5 gene. The forward primer used contained the sequence from base 746–765 in CaMV35S promoter, 5′-CCTTCGCAAGACCC-TTCCTC-3′; the reverse primer was the gene-specific primer 5′-GGATCCTTTATCTGCAC-TTTC-3′ for PV5.

### Southern blot analysis

About 4 $\mu$g of tobacco DNA from each line were digested with 200 U of *Eco*RI restriction enzyme for 26 h in a 200 $\mu$l reaction mix, concentrated and electrophoresed on a 1% (w/v) agarose gel. Southern analysis was performed as described earlier (Yevtushenko et al. 2005). Probe (*EcoRI* and *HindIII* digested fragment of the vector pPV5) was labeled with [$^{32}$P]-dCTP using the random-primed procedure (Invitrogen) and used at $2 \times 10^6$ cpm of purified probe in 1 ml of hybridization solution. Blots were exposed to X-ray film (Kodak BioMax) overnight at –80°C using an intensifying screen. The membrane was also analyzed with a PhosphorImager (Molecular Dynamics, USA).

### Northern blot analysis

Thirty micrograms of total RNA was subjected to electrophoresis on 1% agarose–formaldehyde gel. Accumulation of 10R and 11R transcripts was analysed by northern blot as described earlier (Yevtushenko et al. 2005).

### Antimicrobial activity of PV5

To determine peptides antibacterial activity, 20 $\mu$l (40 $\mu$g ml$^{-1}$) (with final concentration of 8 $\mu$g ml$^{-1}$) was mixed with 80 $\mu$l of a bacterial suspension (grown overnight and diluted 1:25 times in Luria-Bertani (LB) broth) in microtitre plates. Plates were incubated at RT with shaking for 8 h (stationary phase). The inhibitory effect was measured as the change in $A_{630}$ (Beckman DU 60), and the inhibitory effect of peptide was calculated as a function of decrease in absorbance.

The minimum inhibitory concentration of PV5 against plant pathogenic bacteria- *Erwinia carotovora* was determined by inoculating $2 \times 10^5$ bacteria ml$^{-1}$ with different peptide concentrations. After 4 h, this mixture of peptide and bacterial culture was plated on LB agar plates and was incubated overnight at 28°C. Bacterial survival was assayed by colony counts for different concentrations of peptide.

### PV5 cytotoxicity assay

Tobacco protoplasts were isolated from leaves of non-transgenic plants as described by Medgyesy et al. (1980). The effect of PV5 phytotoxicity was observed with an inverted light microscope as described by Yevtushenko et al. (2005).

Leaf discs 11 mm in diameter were cut with the help of a cork borer from the second and third expanded leaves and washed with $dH_2O$. Each disc was weighed, washed twice with $dH_2O$ and then placed in different amounts of peptides ranging from 0–100 $\mu$g or as controls in 24 well plates. The TTC assay for leaf discs and plantlets was performed according to Chang et al. (1999). The samples were centrifuged at 1000*g* and the $A_{530}$ was measured in Beckman DU-65 spectrophotometer. Viability was expressed as $A_{530}$.

The Evans blue assay was performed on leaf discs as described by Baker and Mock (1994). Leaf discs were incubated in different amount of peptides or controls. Evan's blue (0.25% aqueous solution) was added to the incubated discs and these were ground using a glass pestle. Samples were centrifuged at 13,000*g* for 15 min, supernatants collected and $A_{600}$ measured (Beckman DU-65).

### Plant resistance bioassays

Determination of antibacterial activity of detached leaves from transgenic plants was previously described (Osusky et al. 2004). After 7 days incubation, the area of necrotic halos were photographed digitally (Nikon Coolpix 990) and was used as the criterion to evaluate disease resistance in transgenic plants and non-transgenic plants.

Similarly positioned leaves (counted from the bottom) from the same age transgenic tobacco plants, grown in a greenhouse, were detached and placed in Petri plates with moist filter paper discs. Fungal strains were cultured on Potato dextrose agar (PDA; Difco, Detroit, Michigan) medium. Plugs of inoculum were prepared by excising fungal mycelial mats from a culture in a Petri dish using a cork borer and placing the plug on each leaf. The plugs were placed mycelial side down on the adaxial side of each plant in the centre on the mid vein. The inoculated leaves were then placed in diffused light at RT. The extent (area) of the lesion was recorded at an appropriate time for each fungus and the differences in the non-transgenic and transgenic plants recorded digitally as described above.

For comparison of the antiviral activity of the transgenic tobacco plants with non-transgenic plants, leaves were detached from the green house grown tobacco plants and the lesion assay, DAS-ELISA and dot blot hybridization performed as described earlier for in vitro TMV testing.

### Statistical analysis

Statistical analysis of the data was performed with SPSS software (version 12; SPSS Inc, Chicago, IL, USA). ANOVA was used to test for significant differences between different treatments and control, antimicrobial activity of transgenic and non-transgenic plants with a rejection limit of $P > 0.05$.

## Results

In vitro bactericidal activities of peptide at a final concentration of 8 $\mu$g ml$^{-1}$ were tested against different bacteria. This amount is based on the normal range of activity shown by different cationic peptide derivatives in our laboratory. Figure 1A shows significant inhibition of growth of *Erwinia carotovora*, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Escherichia coli* by PV5. These results indicated the broad-spectrum antibacterial activity of PV5. *Fusarium oxysporum* and *Botrytis cineria* were used to test the ability of

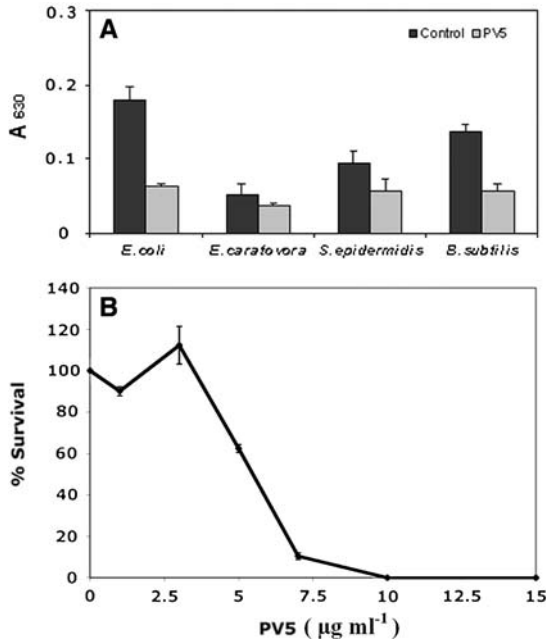


**Fig. 1** **(A)** In vitro bactericidal activities of PV5. *E. coli, E. carotovora, S. epidermidis and B. subtilis* were incubated with the 8 $\mu$g ml$^{-1}$ of PV5 for 4 h as described in ''Materials and Methods'' and growth was scored by measuring $A_{630}$. The asterisk denotes the significant reduction in growth relative to the control at $P < 0.05$ level (Tukey's test), $n = 3$. **(B)** Minimum inhibitory concentration of PV5 on *Erwinia carotovora*. Bacteria were incubated with Peptides (at indicated concentrations) for 4 h, plated, number of colonies counted and the survival of bacteria scored. The results represent the average and standard error of means (SEM) of three independent experiments

PV5 to inhibit fungal growth using a fungal growth zone inhibition assay (Osusky et al. 2005). There was no significant retardation in the growth observed around the discs (data not shown).

The minimal inhibitory concentration (MIC) of PV5 for *Erwinia carotovora* was calculated and defined as the lowest peptide concentration that killed $2 \times 10^5$ bacteria by at least 95%. PV5 is a very potent antibacterial agent as shown by complete bactericidal activity even at low concentration of 10 $\mu$g ml$^{-1}$ (Fig. 1B). This was also observed in in vitro testing of PV5 against different bacteria (Fig. 1A).

A bioassay of plant protoplasts cultivated in liquid medium presents a very sensitive indicator of peptide phytotoxicity. Tobacco mesophyll

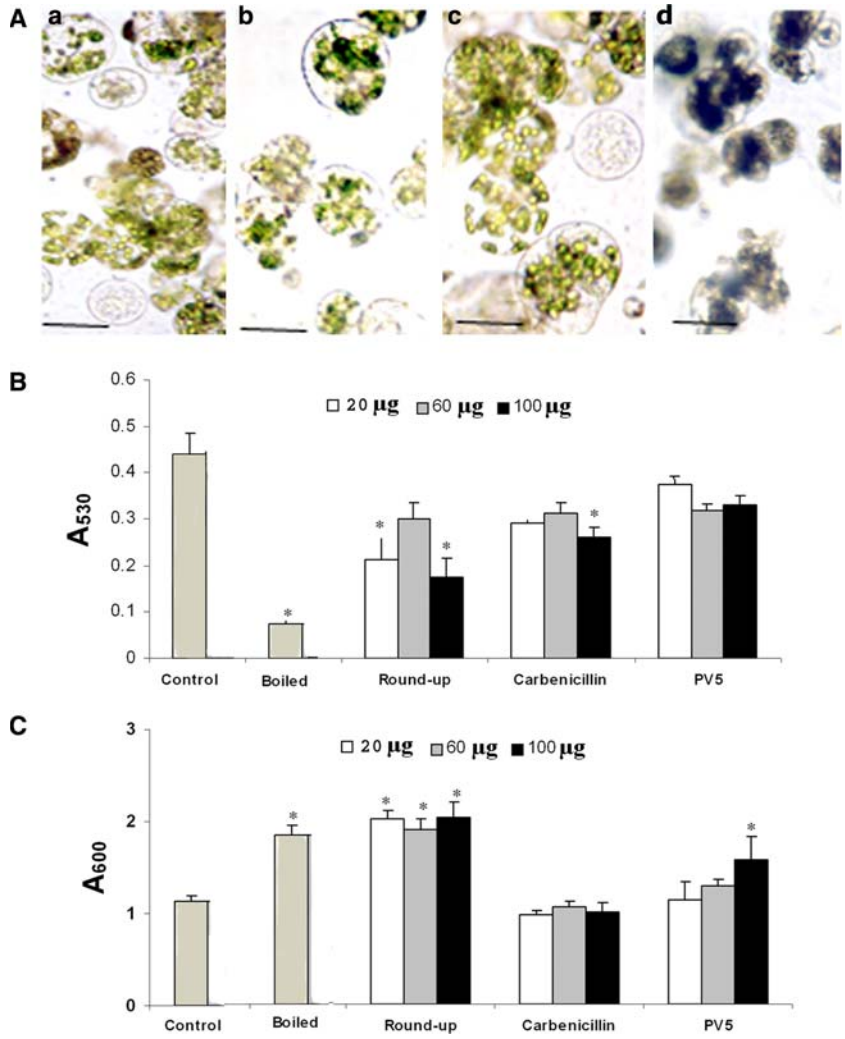


**Fig. 2** **(A)** Effect of synthetic PV5 on the viability of tobacco protoplasts in vitro. Tobacco mesophyll protoplasts were cultivated for 5 days before addition of the peptide. Photographs were taken 24 h after incubation of plant protoplasts (**a**) control (**b**) 10 $\mu$g (**c**) 40 $\mu$g and (**d**) 100 $\mu$g of PV5. Experiment was repeated 3 times. Bar: 50 $\mu$m. **(B)** Effect of different amounts of PV5 on tobacco leaf disc viability as shown by TTC assay **(C)** and Cell death assessed by Evan's blue assay. The sample treated with $dH_2O$ only was used as a control. Round-up® was used as a positive control. Carbenicillin was used as a negative control. Means ± S.E.M ($n$ = 3) are indicated. The asterisk denotes the significant difference from control at $P < 0.05$ (Tukey's test)

protoplasts were cultivated with different amounts of PV5 ranging from 0–100 $\mu$g. The PV5 treatment showed a cytotoxic effect at 100 $\mu$g of peptide as seen by a change of cell shape and color with the dark cytoplasm, indicating cell death (Fig. 2A (d)) relative to the control (not treated with PV5), No visible effect on protoplast viability or cell division were observed below this amount.

A time course study was performed to determine the optimal incubation time for TTC and Evans blue assays for leaf discs (Bhargava 2005). Twelve hours was the appropriate incubation time determined for the TTC assay and Evan's blue assay to assess the effect of peptides on viability (data not shown). Using TTC assays (Fig. 2B) no significant difference was seen in the viability of the leaf disc samples incubated for 12 h in 20 $\mu$g of PV5 as compared to control ($dH_2O$ treated samples). PV5 showed significant reduction in absorbance at 100 $\mu$g of peptide. The significant reduction in viability relative to control (non-treated) in boiled samples and Round-up® treated leaf discs shows the reliability of this assay for assessment of viability of plant leaf discs. In the Evans blue assay, absorbance was directly proportional to the toxicity of the compound. Figure 2C shows a significant increase in absorbance relative to control with Round-up® and boiled samples (shown by asterisk) and illustrates the reliability of the method to assess the cytotoxicity of these peptides on leaf discs. Leaf discs

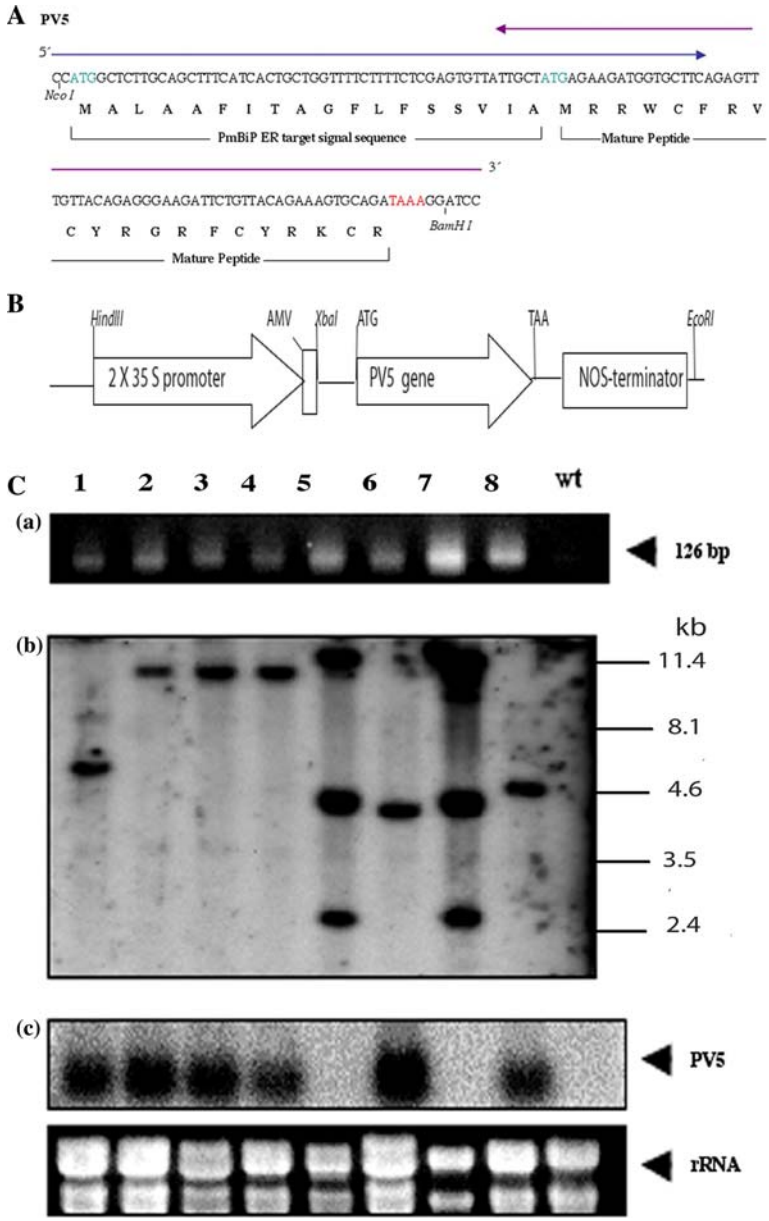


**Fig. 3** **(A)** A schematic presentation of PV5 gene construction. Restriction enzymes sites are indicated in italics. Arrows indicate the positions for the overlapping PCR primers used for gene synthesis. **(B)** The schematic outline of plant expression vector for cationic peptide cDNA. 2 × 35S, duplicated enhancer CaMV 35S promoter; AMV, leader sequence from alfalfa mosaic virus RNA4. **(C)** PV5 gene integration and expression. (**a**) The cationic peptide coding sequence was PCR amplified from DNA isolated from control (non-transformed, lane wt) and transgenic (lanes 1–8: transgenic tobacco lines #1, #5, #6, #7, #8, #9, #11 and #19. (**b**) Southern blot analysis of transgenic plants. Tobacco leaf DNA was digested with *EcoR I*, electrophoresed and hybridized with a $^{32}$P-labelled PV5 probe. The number of bands reflects the number of transgene insertions. For lane designation see (a). (**c**) Northern blot analysis of PV5 mRNA accumulation in tobacco. Total RNA was prepared from leaves of transgenic plants; RNA samples (30 $\mu$g each) were separated by denaturing formaldehyde agarose gel electrophoresis, blotted and hybridized with a $^{32}$P-labelled PV5 probe. Ethidium bromide stained ribosomal RNA bands (rRNA) are shown as loading control

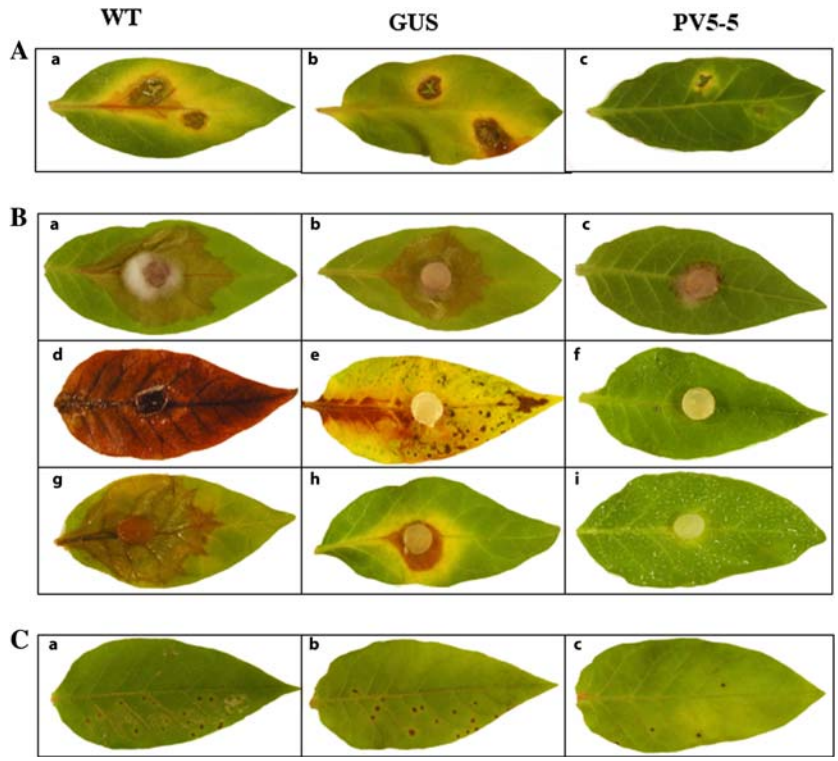


**Fig. 4** Resistance of transgenic tobacco expressing PV5 to phytopathogens. Detached leaves from mature nontransgenic tobacco control, GUS and transgenic tobacco expressing PV5 (line #5) were infected with (**A**) *Erwinia carotovora* (**B**) Fungi: (**a–c**) *Botrytis cineria*, (**d–f**) *Verticillium* sp. (**g–i**) *Fusarium oxysporum* and (**C**) TMV as described in ''Materials and Methods''

treated with 100 $\mu$g of PV5 showed a significant increase in absorbance showing cytotoxicity at this high peptide amount.

In all transgenic tobacco plants, a band of the expected size of 110 bp was seen by PCR (Fig. 3C (a)). Southern analyses of transgenic tobacco plants were performed to determine the stable transgene integration and copy number while northern blot analysis to confirm gene expression. Southern analysis indicated that one to five copies of the transgene were maintained in the plant genome (Fig. 3C (b)). A single copy of PV5 was present in line # 5, # 6, # 7, # 9 and # 19. Multiple copies of genes were integrated in line # 1, # 8 and # 11. The accumulation of the PV5 transcript was determined by northern blot analysis of leaf RNA. The results showed PV5 expression in lines, # 5, # 6, # 7, # 9 and # 19. However, line # 8 and # 11 (lane 5 and 7) with multiple gene copies showed no expression thus illustrating the correlation between high copy number and gene silencing or positional effect (Fig. 3C (c)).

For in planta antibacterial testing, leaves from transgenic and non-transgenic plants were wounded and inoculated with 10 $\mu$l (~$5 \times 10^5$ cfu) of an *E. carotovora* suspension. All of the transgenic lines tested had a single gene copy number but significant expression levels. After 6 d, the extent of decay was much higher in the control plants as compared to the transgenic plants. Figure 4A shows the transgenic PV5 (line # 5) expressing plants resistant to *Erwinia*. The experiment was performed three times and similar pattern of resistance was observed.

To study the response of PV5-expressing tobacco plants to fungal infection, detached leaf fungal assays were performed. Figure 4B shows the results of the fungal infection assays on control (non-transgenic and transgenic GUS control) and a transgenic line with single copy of the gene PV5 # 5. *Botrytis cineria*, *Verticillium sp*. and *Fusarium oxysporum* (Fig. 4B) were inoculated on the detached leaves. Six to 20 days after infection the leaves from the non-transgenic tobacco and transgenic control (GUS) were heavily infected by *Botrytis*, *Verticillium* and *Fusarium* infection. In contrast, infected areas on the transgenic leaves, independent of the gene

copy number, were much smaller and limited to the area around the site of contact with the fungus containing agar plugs (Fig. 4B).

TMV lesions on transgenic leaves were counted and compared with the controls (non-transgenic plants) (Fig. 4C). A significant reduction in the number of lesions in transgenic plants was observed (Fig. 4C). Similar results were seen by DAS-ELISA of extracts from the TMV infected leaves (Fig. 5C). Transgenic plants showed decrease in the TMV propagation as seen in Fig. 5A, that shows the dot blot hybridization with a TMV movement protein cDNA. Further quantification of $^{32}P$ radioactive count of hybridization signal was performed. Figure 5B shows the significant reduction in the CPM and thus the reduction in the extent of TMV infection in transgenic tobacco plants expressing PV5.

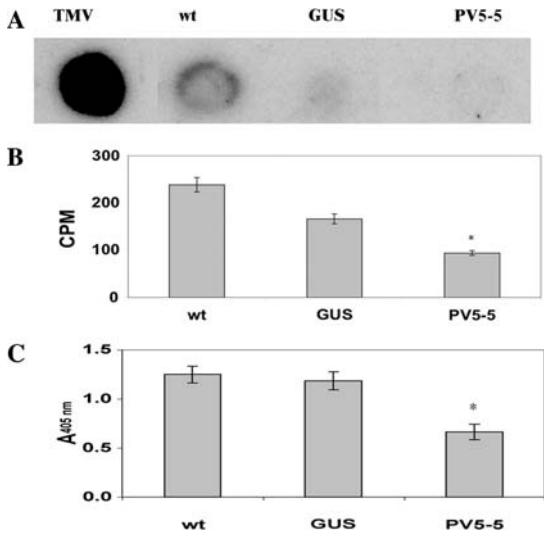


**Fig. 5** TMV resistance in transgenic plants. (**A**) Comparison of TMV infection in transgenic and control tobacco plants leaves by Dot blot hybridization (**B**) CPM count of the quantitative dot-blot hybridization with TMV movement protein (MP)-cDNA probe and TMV infected PV5 expressing plants. Detached leaves were infected with TMV. Dot-blot was performed as described in ''Materials and Methods'' and extent of hybridization was done by assaying $^{32}P$ radioactivity. (**C**) DAS-ELISA based TMV resistance shown by transgenic tobacco plants expressing PV5. Detached leaves were infected with TMV. ELISA was performed as described in ''Materials and Methods''. $A_{405}$ represents the DAS-ELISA absorbance values. Error bars show SEM, $n = 3$. Significant differences between mean values of controls (non-transgenic and GUS) and transgenic tobacco plants are indicated by * at $P < 0.05$ (Tukey test)

## Discussion

The MIC of PV5 for *E. carotovora* was 2–4 times higher than that of other peptides described earlier (Osusky et al. 2000, 2005). PV5 showed no in vitro antifungal activity against *Verticillium* and *Fusarium* strains, thus indicating poor antifungal property towards these fungi. This is in agreement with the earlier reports showing polyphemusin to be more antibacterial and antiviral (Arakaki et al. 1999; Murakami et al. 1991; Zhang et al. 2000; Robinson et al. 1998) than antifungal in nature. There are no reports describing in vitro antiviral activity of a polyphemusin against plant viruses.

Synthetic derivatives of polyphemusin have been shown to have low cytotoxicity to animal cell lines and higher toxicity to microorganisms (Zhang et al. 2000), which makes them good candidates for expression in plants. Our results using leaf discs showed that PV5 exhibited low cytotoxicity even up to 60 $\mu$g. In previous studies, TTC assays were successfully employed to predict the germinability of seeds and viability of plant tissues exposed to stressful conditions and injury (Chang et al. 1999; Steponkus and Lanphear 1967; Towill and Mazur 1974). The parallel in the relative changes between TTC reduction activity in the leaf discs and Evan's blue staining, suggest that these assays could be used to determine the cytotoxic effects of peptides and other toxic compounds against plant leaf tissues. Use of carbenicillin as the negative control was based on the hypothesis that there would be no effect of this antibiotic against leaf discs, protoplasts as well as plantlets. Carbenicillin has been extensively used at high concentrations (up to 500 $\mu$g) in plant tissue culture and found to be safe on plant calli and shoots during transformation and selection processes. Together these tests provide a set of indicators of cytotoxicity and can readily be adapted for mass screening of peptide(s) and their variants, particularly in association with combinatorial chemistry.

In protoplasts, PV5 at 100 $\mu$g was cytotoxic when observed by light microscopy (Fig. 2A). Similar cytotoxicity at high amounts was reported earlier with cecropin and melittin (Mills and Hammerschlag 1993; Yevtushenko et al. 2005).

The amount of peptide that affected plant cell viability was about 10-fold higher than required to inhibit plant pathogenic bacteria completely (Fig. 1B). These tested and non-toxic amounts (20 and 40 $\mu$g) are much higher than the amount likely to be expressed in transgenic plants. Earlier it had been shown that plants with 2 × 35S CaMV promoter express cationic peptides in the range of 1–5 $\mu$g/g of fresh tissue weight (Carmona et al. 1993; Osusky et al. 2000, 2005). At 100 $\mu$g, an amount at which PV5 was cytotoxic is very high and it is highly unlikely that PV5 would reach this amount in transgenic plants. All the generated transgenic lines expressing PV5 did not show any difference in the morphology when grown in the green house (data not shown) and thus indicated no phytotoxic effect of these peptides in plants.

In planta studies showed that tobacco plants expressing PV5 are resistant to a plant pathogenic virus, bacteria and fungi. These results are similar to recent works using synthetic variants and chimeras of other cationic peptides for conferring broad-spectrum bacterial and fungal resistance in plants (Gao et al. 2000; Osusky et al. 2000, 2004, 2005; Yevtushenko et al. 2005). Earlier, expression of tachyplesin along with a signal sequence in plants has been shown to confer resistance against *Erwinia* soft rot (Allefs et al. 1996). PV5 is the isopeptide of tachyplesin and similar antibacterial resistance was expected with polyphemusin expression in tobacco.

The signal sequence of Douglas-fir (*Pseudotsuga menziesii*) luminal binding protein (BiP) (Forward et al. 2002) in conjuction with the 2 × 35S and AMV promoter was used to target the antimicrobial peptide (CAPs) to extracellular spaces via the default secretory pathway. This BiP signal sequence was included in polyphemusin constructs of PV5 (Fig. 3A) in order to enhance the yield of the peptide, stability, and function. Accumulation in the intercellular spaces may also prevent phytotoxic effects of CAP expression, and allow contact of the peptides with invading pathogens before they enter the cell sequence (Turrini et al. 2004). Levels of cecropin B transcript in transgenic tobacco increased 4- to 5-fold when peptide's native N-terminal secretory signal was incorporated. Furthermore, substitution of the insect secretory signal with a plant-derived sequence increased cecropin B transcripts 10- to 12-fold (Florack et al. 1995). Commonly used plant secretory signals include those derived from pea vicilin protein and the tobacco PR-1a and PR-1b pathogenesis related proteins. In this study we used a Douglas fir BiP protein signal sequence to direct CAPs to tobacco apoplast. However, based on results, the effectiveness of this strategy remained inconclusive.

The effects of genomic position and copy number on transgene expression have been reported in plants (Allen et al. 2000). The transgenes integrate at random sites during transformation. Some integration may occur in transcriptionally active chromatin environments, others in transcriptionally inert chromatin regions (Mengiste et al. 1999). It is believed that transgenes in heterochromatic areas are prone to silencing and give rise to reduced and/or variable expression (Allen et al. 2000; Mengiste et al. 1999). Figure 3C (c) shows no expression in line # 8 and line # 11 (lane 5 and 7), which could reflect this positional insertion effect. Another reason for a lack of expression could be gene silencing. Plants with multiple copies of the transgene and/or high levels of transgene transcription are more likely to exhibit gene silencing than plants with a single copy and low-level transcription (Flavell 1994). There are conflicting reports about the relationship between copy number and expression level (Hobbs et al. 1993).

PV5 provided resistance against *Fusarium*, *Botrytis* and *Verticillium* when expressed in planta but it was not apparent in vitro. This indicates not only an enhanced disease resistance in transgenic plants due to cationic peptide expression but also interaction of the expressed peptides with other unknown plant components to enhance the resistance against plant pathogens. Overexpression of certain factors shown to be involved in the immune responses has produced disease resistance in plants (Cao et al. 1998). Expression of cationic peptides in plants can trigger the defense signaling or enhance the innate immunity in plants. In addition to direct antimicrobial activity, there are reports of the role of cationic peptides in regulation of innate immunity and defense responses in living organisms (Chernysh et al. 2002; Hancock and Diamond 2000). The

question as to whether these antimicrobial peptides modulate gene expression or provides cross-talk with other defense signaling pathways is not clear. In the present study, there was an indication of enhancement in disease resistance against fungi and TMV in transgenic plants. Therefore, it is possible that the expression of the cationic peptides in plants modulates defense gene expression or provides cross talk with other defense signaling pathways.

Successful application of a transgenic approach using cationic peptides to control plant diseases, including viruses, will likely help eradicate plant diseases. It will also reduce the environmental impact of intensive use of pesticides and improve the quality of our food.

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