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PLANT SCIENCE
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Antiviral indolicidin variant peptides: Evaluation for broad-spectrum disease resistance in transgenic *Nicotiana tabacum*

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Received 22 June 2006; received in revised form 2 October 2006; accepted 30 October 2006
Available online 8 December 2006

Abstract

Cationic peptides play an important role in the natural defenses against microbial infections of most of the living organisms. Indolicidin, a cationic peptide derived from bovine neutrophils, demonstrates *in vitro* antibacterial, antifungal and antiviral activity against animal pathogens. To evaluate efficacy of this peptide aganist plant pathogens, especially viruses, here we describe engineering transgenic plants, expressing indolicidin variants (10R and 11R). The variants were tested *in vitro*, for their effectiveness against plant pathogenic bacteria, fungi and TMV. Genes encoding these peptides were introduced into the tobacco cultivar (*Nicotiana tabacum* var. Xanthi). Leaf assays of transgenic plants showed enhanced resistance against several pathogens including significant resistance against TMV challenge. This is the first report of heterologous cationic peptide mediated antiviral resistance in engineered plants.
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Keywords: Indolicidin; Tobacco; Antiviral peptide; Transgenic

1. Introduction

Cationic antimicrobial peptides have been found in a variety of sources, from prokaryotes to higher eukaryotes and constitute part of the first line of host defense [1,2]. In the past 25 years, more than 800 cationic, gene-encoded antimicrobial peptides have been described and majority (96%) has a net positive charge. The mode of action of cationic peptides is not completely known; however, specificity with regards to the pathogen as well as the peptide has been demonstrated. The prokaryotic membranes are composed of negatively charged phospholipids and cationic peptides function by interacting with the cytoplasmic membrane of the bacteria and disrupting them [3]. The eukaryotic cell membrane is largely composed of zwitterionic lipids contributing to the membrane selectivity of cationic peptides between prokaryotic and eukaryotic cells.

Indolicidin, a 13-amino acid long cationic peptide, rich in tryptophan and proline, is present in the cytoplasmic granules of bovine neutrophils [4]. As a smallest naturally occurring linear antimicrobial peptide, indolicidin has a unique composition consisting of 39% tryptophan and 23% proline with amino acid sequence of ILPWKWPWWPWRR. Indolicidin has shown *in vitro* inhibitory activity against Gram-negative and Gram-positive bacteria [4–6], protozoa [7], fungi [8] and viruses [9,10]. In addition, the peptide is cytotoxic to rat and human T lymphocytes and lyses erythrocytes. The development and characterization of indolicidin analogues on the basis of design, mechanism of action and reduced toxicity [5] resulted in molecules, exhibiting reduced toxicity to erythrocytes and increased broad-spectrum antimicrobial activity. *In this study*, two new indolicidin synthetic variants (10R and 11R) were selected as model peptides to tap into and exploit the potential of these cationic peptides to engineer plant pathogen resistance, including viruses.

Non-conventional strategies for the production of disease-resistant crop plants have employed gene transfer technology for molecular resistance breeding [11,12]. Such strategies have included expression of plant defense response pathway

Abbreviations: MS, murashige-skoog; PDR, pathogen derived resistance; TMV, tobacco mosaic virus; DAS-ELISA, double antibody sandwiched ELISA

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doi:10.1016/j.plantsci.2006.10.016

components [13]; expression of genes encoding hydrolytic enzymes [14,15]; elicitors of defense response [16]; and genes encoding small peptides [17,18]. Recently indolicidin variants showed enhanced resistance to *Erwinia* when expressed in tobacco [19].

Resistance to plant viruses can be conferred either by expressing part of the viral genome or virally associated sequences, popularly known as pathogen-derived resistance (PDR). Strategies for PDR are divided into those that require the production of protein and those that require only the accumulation of the viral nucleic acid sequences. The capsid protein, antisense RNA and truncated replicase protein have been used for generation of transgenic plants for resistance to viruses [20–22]. However, viral resistance provided by PDR is very specific and there are concerns about risks of releasing transgenic plants containing viral sequences in the field, Novel sources of resistance have been introduced in plants to overcome the problem of PDR [23,24]. Recently, a novel approach using protein-mediated resistance to provide broad virus resistance was shown by Rudolph et al. [25]. Expression of antimicrobial peptides and their synthetic variants in plants for broad-spectrum disease resistance have shown promising results [11,19,26–31]. However, so far resistance to viruses has not been reported. Here, we demonstrate antiviral activity of indolicidin peptides against a plant virus and promising approach for engineering broad-spectrum disease resistance including viruses in engineered plants. *In vitro* assays using 10R and 11R showed a broad-spectrum activity against plant pathogenic bacteria and a virus. Assessment of *in planta* activity was performed by constitutively expressing these cationic peptides in transgenic tobacco. The disease assays on transgenic tobacco plants showed a broad-spectrum disease resistance against bacteria, fungi and TMV.

2. Materials and methods

2.1. Peptides

Peptides used in this study were the synthetic indolicidin derivatives, 10R (MRRPWKWPWWPWRR) and 11R (MRWKKWPWWPWRRK). Peptides were synthesized using a model 430 A peptide synthesizer (Applied Biosystems Inc, Foster City, CA) with the 0.25 mmol scale FastMoc chemistry software at the University of Victoria Genome BC Proteomics centre. Lyophilized peptide powders were reconstituted in sterile, deionized water to a stock concentration of 1 mg ml^{-1}, filtered though a 0.2 μm Millipore filter and stored at −20 °C until further use.

2.2. Plant material

Leaves of non-transgenic tobacco plants (*Nicotiana tabacum* var. Xanthi) were used for transformation. With the exception of T_0 all wild type and transgenic plants were germinated from seeds at the same time. The only difference in growth media was the presence of kanamycin for selection. All plants and leaves were of the same age. T_0 controls were germinated on MS media with no hormone. With this exception all experimental procedures and plant materials were identical in both transgenic and control plants. Selected T_1 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).

2.3. Pathogen cultures

Bacterial strains used in this study were the laboratory isolate of *Erwinia carotovora*. Fungal pathogens used were *Botrytis cinerea* and *Verticillium* sp. obtained from Simon Fraser University, Burnaby. The plant virus used was tobacco mosaic virus (TMV) (ATCC 135 PV) obtained from American Type Culture Collection, Atlanta.

2.4. In vitro antibacterial activity

The minimum inhibitory concentration of peptide derivatives against plant pathogenic bacteria—*Erwinia carotovora* was determined by incubation of 2×10^5bacteria 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.

2.5. Plant virus assays

2.5.1. Lesion assay

For assessing the *in vitro* antiviral activity of cationic peptides, detached leaves of similar age, non-transgenic tobacco plants grown in a green house, were used. Half-leaves were randomly selected in each assay. The lesion assay was performed as described [32] with some modifications. Different amounts of cationic peptide variants were mixed with active fixed amount (5 μg) of virus samples. Controls had only viruses and buffer with no peptides. Ten microlitres of sample and control were applied to each half of the detached leaves. Leaves were rinsed with water and placed on dH_2O soaked filter paper in 6″ Petri dishes. The samples were then moved to a growth chamber (photoperiod of 12 h, 25 °C) where they were monitored daily for appearance of symptoms. Lesions of 1–2 mm in diameter were counted after 3 days. Numbers of lesions were compared with the control. Infected leaf halves and non-infected halves (control for each sample) were collected 4 days after inoculation and each half (sample and control) was homogenized and stored at −20 °C.

2.5.2. Double antibody sandwiched ELISA (DAS-ELISA) for TMV

Leaf extracts from systemically infected, collected and stored (−20 °C) tobacco leaf halves (control and infected) from the lesion experiments were obtained by grinding leaf tissues in liquid nitrogen. The powder was incubated on ice for 10 min and then the leaf sap was collected by microcentrifugation at 13,000 × *g* for 25 min. Supernatant was collected in a fresh

tube and frozen at −80 °C until further use in DAS-ELISA and dot blot hybridization.

Viral infection quantitation between control and infected samples was performed by double antibody sandwich ELISA with a commercial TMV detection kit (Agdia Inc., Indiana, USA). One hundred microlitres of 10 times diluted sap was used for ELISA. Tests were performed according to the manufacturer's instructions. Absorbance at 405 nm was determined with the aid of an automated microplate ELISA reader (Bio-Tek, Burlington, VT). A reaction was considered affirmative if the absorbance was high in the positive control provided by the manufacturer.

2.5.3. Dot blot hybridization for TMV quantitation

A piece of Biodyne B nylon membrane (Pall) was cut and marked into 1 cm × 1 cm squares with a pencil. Leaf extracts (5 μl) were spotted on to the marked squares. Membranes were then baked at 80 °C under vacuum for 1.5 h. Movement protein cDNA, cloned earlier was used as a probe. It was labeled with [^{32}P]-dCTP using the random-primed procedure (Invitrogen) and used at 4×10^6 cpm of purified probe in 1 ml of hybridization solution. Pre-hybridization and hybridization steps were carried out at 65 °C for 2 h and 16 h, respectively in PerfectHyb™ Plus buffer (Sigma) according to the manufacturer's protocol. Blots were then incubated in hybridization bottles containing 0.1% SSC and 0.5% SDS solution for 20 min at 65 °C followed by final wash in 0.5% SSC, 0.1% SDS at 65 °C for 10 min, and then exposed to X-ray film (Kodak BioMax) overnight at −80 °C using an intensifying screen.

Dot blot signal quantification was done by measuring cpm for each dot. Each square was cut and was suspended in scintillation cocktail, and then radioactivity was measured in Beckman (LS 5000CE) scintillation counter.

2.6. Construction of cationic peptide genes and DNA manipulation

Gene specific overlapping synthetic oligonucleotides were designed for construction of 10R and 11R genes (Fig. 1B). These were based on the nucleotide sequences of the two peptide variants, determined by reverse-translation of the amino acid sequence using plant specific codons and modifying them by adding specific restriction enzyme sites for DNA cloning and expression. The genes were assembled by PCR using these oligonucleotides. DNA manipulations were carried out as described [33]. The enzymes were purchased from Amersham Pharmacia Biotech (England) or New England Biolabs (Beverly, MA, USA) and used according to manufacturer's instructions.

Transformation of Agrobacterium tumefaciens MP90 was done by the freeze–thaw method [34]. Transformation of tobacco plants was carried out as described by Horsch et al. [35].

2.7. Genomic DNA and total RNA isolation from plant tissue

Total Genomic DNA was extracted from the leaves of T_1 and control plants using a GenElute™ Plant Genomic kit (Sigma) according to the manufacturer's instructions. Total leaf RNA was prepared using TRIzol (GIBCO BRL, Rockville, MD, USA) following the manufacturer's protocol.

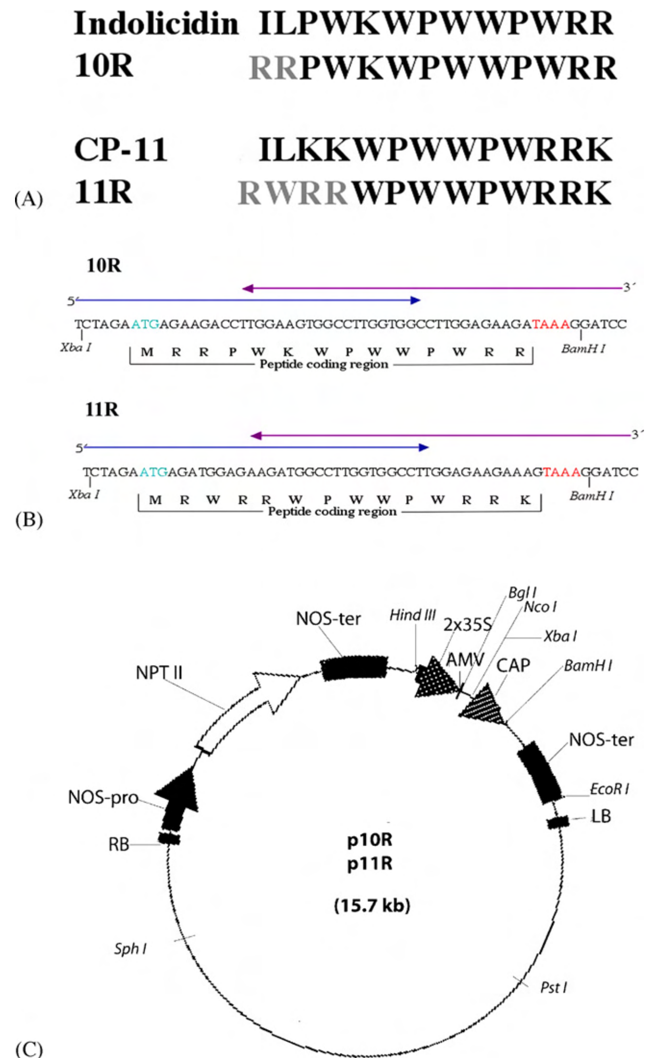

Fig. 1. (A) Amino acid sequences of 10R and 11R variants compared with their parent sequences. The substituted residues in 10R and 11R are shown in light. (B) A schematic presentation of 10R and 11R gene construction. Restriction enzymes sites are indicated in italics. Arrows indicate the positions for the overlapping PCR primers used for gene synthesis. (C) The schematic outline of plant expression vector for cationic peptide cDNA. The abbreviations in the figure are as follows: RB and LB, the right and left border regions of the Ti plasmid; NOS-pro and NOS-ter, promoter and terminator respectively, of the nopaline synthase gene; NPT II, neomycin phosphotransferase II; 2× 35S, duplicated enhancer CaMV 35S promoter; AMV, leader sequence from alfalfa mosaic virus RNA4; CAP, protein coding sequence of either 10R or 11R.

2.8. PCR analysis of transgenic plants

PCR was carried out in 50 μl of reaction mixture containing 200 ng of plant DNA, Taq PCR Master Mix (Qiagen, Mississauga, Ontario, Canada) 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 10R and 11R constructs that amplified the

appropriate genes were: forward primer, the primer with sequence from base 746–765 in CaMV35S promoter 5′-CCTTCGCAAGACCCTTCCTC-3′, and reverse primers were gene specific primers 5′-GGATCCTTTATCTTCTCC-3′ for 10R and 5′-GGATCCT TTACTTTCTTC-3′ for 11R.

2.9. Southern analysis

Four micrograms of tobacco DNA from each independent line were digested with 200 U of *Eco*RI restriction enzyme for 26 h in a 200 μl reaction mix, concentrated and electrophoresed on a 1% (w/v) agarose gel. Southern analysis was performed as described earlier [31]. Probes (*Eco*RI and *Hin*dIII digested fragment of the vector p10R or p11R) were labeled with [^{32}P]-dCTP using the random-primed procedure (Invitrogen) and used at 2×10^6 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).

2.10. 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 [31].

2.11. In planta disease assays

Transgenic (T_1) and non-transgenic tobacco plants were grown in a greenhouse at 26 °C under natural and artificial light (15/9 h photoperiod, 200 mE m^{-2} s^{-1}). Leaves were collected from two-month-old (6–8 developed leaves) plants. Similarly positioned leaves (counted from bottom) from same aged plants were detached and placed in Petri plates with moist filter paper discs. For each trial, two inoculation with three replicates arranged in a randomized manner were performed. To exclude the possibility that increased resistance of transgenic plants is triggered by the transformation event; control plants transformed with the vector pBI 121 [expressing GUS with 35S promoter and an untranslated leader sequence from alfalfa mosaic virus (AMV) RNA4 that acts as a cis active ‘‘translational activator’’] were included in all disease assays.

2.11.1. Antibacterial resistance of detached leaves from transgenic plants

Overnight culture of bacteria was diluted to a concentration of about 1×10^7 cells ml^{-1}. An aliquot of 10 μl of this suspension was applied onto a tobacco leaf surface followed by piercing with needle as a way of inoculating bacteria into the leaf. The inoculated leaves were kept at room temperature. After 7 days of incubation, necrotic lesions had formed around the inoculated points. The comparison of area of the necrotic lesions was the criterion used to evaluate disease resistance in transgenic plants as compared to non-transgenic plants. Pictures were taken by digital camera (Nikon Coolpix 990).

2.11.2. Antifungal resistance of detached leaves from transgenic plants

Fungal strains were cultured on potato dextrose agar (PDA; Difco, Detroit, Mich. Plugs of inoculum were prepared by cutting fungal mycelium with a cork borer from culture in a Petri dish and placing it on each leaf. The plugs were placed mycelial side down on adaxial side of each plant in the centre on the mid vein. The inoculated leaves were then incubated in diffused light at room temperature. The extent of lesion was recorded at appropriate time and the difference in the non-transgenic and transgenic plants was observed. Pictures were taken with a digital camera (Nikon Coolpix 900).

2.11.3. Antiviral resistance of detached leaves of transgenic plants

For comparing the antiviral activity of the transgenic tobacco plants with non-transgenic plants, leaves were detached from the green house grown tobacco plants and lesion assay was performed as described above for *in vitro* TMV testing. The number of lesions was counted on transgenic plants and compared with the lesions on non-transgenic plants. Each half (sample and control) leaf was homogenized and stored at −20 °C. DAS-ELISA and dot blot hybridization was performed as described earlier.

2.12. 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 $P > 0.05$.

3. Results

3.1. Indolicidin variants: 10R and 11R

Indolicidin is one of the smallest natural antimicrobial peptides. Earlier attempts to increase charge in the native peptide have resulted in enhanced antimicrobial activity [5,36]. The design of 10R and 11R was also based on increasing the number of positively charged residues in specific positions, while maintaining the overall length (net charge of native indolicidin is +3, 10R is +4 and 11R is +5). Indolicidin was modified to create 10R by replacing non-polar amino acids leucine and isoleucine at N-terminus by arginines (Fig. 1A). 11R was similar to CP-11, a indolicidin variant described earlier [37], except that a tryptophan residue was added at N-terminus by replacing one arginine and the two lysines at N-terminus in the peptide sequence were replaced by arginines (Fig. 1A).

3.2. 10R and 11R have broad-spectrum in vitro antimicrobial activity

The minimal inhibitory concentration (MIC) of 10R and 11R for *Erwinia carotovora* was calculated and defined as the

lowest peptide concentration that killed 2×10^5 bacteria by at least 95%. 11R and 10R showed complete bactericidal activity at concentrations of 10 μg ml^{-1} and 40 μg ml^{-1} respectively (Fig. 2). 11R showed bactericidal activity at a much lower concentration than 10R.

Indolicidin has shown *in vitro* antiviral activity against human immunodeficiency virus (HIV) and herpes simplex virus (HSV) [9]. However, there was no report of testing these peptides and their variants on plant viruses. Therefore activity of these peptides on tobacco mosaic virus was determined. The results of lesion count indicated a significant reduction in lesion development (data not shown). DAS-ELISA is a sensitive method to detect and quntitate virus infection in plants. Fig. 3C shows the reduction in TMV infectivity in tobacco plants after peptide treatment as determined by DAS-ELISA. The results obtained from dot blot hybridization with the TMV movement protein cDNA probe are in agreement with the ELISA results (Fig. 3A). In this case, instead of antibodies, a ^{32}P-labelled cDNA probe was used to assess the extent of TMV infection and the amount of TMV RNA present. To further confirm the reliability of this hybridization and extent of infection, the dots were excised and the radioactivity was measured for each sample. Fig. 3B shows the reduced radioactivity count in the peptides treated samples and hence the reduction in the extent of TMV infection. The Lesion assay, DAS-ELISA and dot blot hybridization together all show antiviral activity of 10R and 11R peptides against TMV. This also signifies the *in vitro* inhibition in TMV propagation by 10R at amounts as low as 10 μg.

3.3. Plant expression vectors p10R and p11R

The genes for cationic peptides were assembled using the polymerase chain reaction (Fig. 1B) and the expression vectors were constructed. Genes for 10R and 11R had *Xba*I and *Bam*HI sites at 5′ and 3′ ends. These were first cloned in to vector PBI 524 [containing a duplicated enhancer cauliflower mosaic virus (CaMV) 35S promoter and an untranslated leader sequence from alfalfa mosaic virus (AMV) RNA4, that acts as a cis active

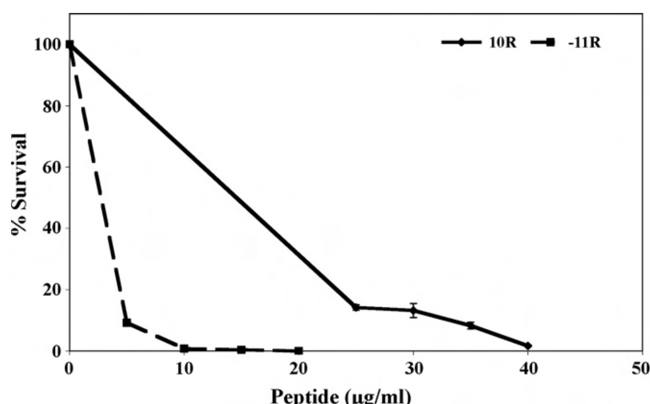

Fig. 2. Minimum inhibitory concentration of 10R and 11R on *Erwinia carotovora*. Bacteria were incubated with Peptides (at indicated concentrations) for 4 h, plated, number of colonies counted and the survival of bacteria was scored. Results represent the average and standard error of means (S.E.M.) of three independent experiments. Three repeats in each experiment were performed.

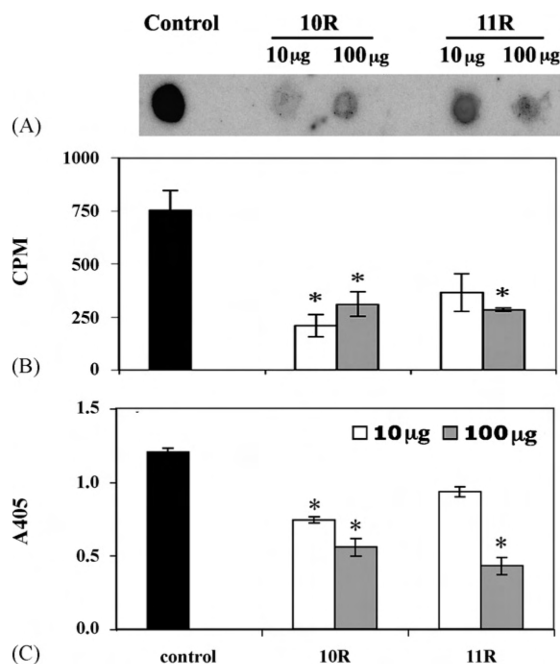

Fig. 3. Effect of 10R and 11R on infectivity of TMV. (A) Dot-blot hybridization of TMV movement protein (MP)-cDNA probe with 10R and 11R treated TMV and infected samples. (B) CPM counts for anti-TMV activity. The extent of hybridization was confirmed by ^{32}P radioactivity assay. (C) DAS-ELISA showing decrease in TMV propagation after treatment with 10R and 11R. TMV was mixed with 10 μg and 100 μg of each peptide, incubated for 24 h at room temperature and inoculated onto 3 or 4 tobacco leaves. TMV was detected in leaf extract of TMV infected leaf halves by DAS-ELISA. Leaves were either infected with peptide treated TMV or non-treated (control) and incubated at 25 °C for 5 days. Hundred microlitre of 1:10 diluted leaf extract was used for TMV detection. Results shown are the average of three independent experiments. The asterisk denotes the significant reduction in growth relative to the control at $p < 0.05$ (Tukey test).

‘‘translational activator’’] and later digested with *Hin*dIII and *Eco*RI enzymes. This *Hin*dIII–*Eco*RI fragment, containing a 2 X 35S promoter with an AMV RNA4 translation-enhancing element and 10R and 11R with a NOS terminator (NOS-ter) was ligated in to *Hin*dIII–*Eco*RI digested pBI 121 vector (thus replacing the 35S promoter and the β-glucuronidase (GUS) gene with the NOS-terminator)(Fig. 1C).

3.4. 10R and 11R are integrated and transcribed in transformed plants

High regeneration with multiple shoots per explant was achieved for tobacco after transformation on the selective medium (100 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin). Putative transgenic shoots were randomly selected and rooted in the presence of selective agent (kanamycin 500 mg l^{-1}). All plants retained the normal morphology for tobacco with no indication of cytotoxicity due to the expression of 10R or 11R peptides in T_0 and T_1 generation. T_0 plants were analyzed with PCR and selected independent lines were transferred to green house. T_1 seeds germinated in the presence of kanamycin produced green seedlings whereas control seedlings were bleached. Six independent lines were selected and T_1 transgenic plants for each line were analyzed for gene integration by PCR

and Southern blot and expression by Northern blot (Fig. 4). PCR analysis using upstream primers specific for the 2× 35S-promoter and downstream primers specific to either the cationic peptide gene (10R and 11R) was performed (described in Section 2). DNA from the untransformed plant of similar age was used as a negative control (wt). In 10R and 11R plants, a band of the expected size of 60 bp was observed (Fig. 4A). Southern analysis with 10R showed a single copy of 10R in lines #4, #6 and #21. Multiple copies were detected in Line #11, #19 and #20 [Fig. 4 (i B)]. The expression was low in #4 and #20 [Fig. 4 (i C))]. Southern blot of 11R showed Lines #1 and #16 with single gene copy. Lines #7, #12, #13 and #17 showed multiple gene copy integration and high expression was seen in all lines of 11R plants [Fig. 4 ii (B) and ii (C)].

3.5. Resistance of transgenic plants to bacterial, fungal and viral infection

Pathogen testings were performed on all the generated and tested independent lines (T_1). In this report, we have shown data for the representative lines with single gene copy and high expression levels for both 10R (Line #6) and 11R (Line #16). There was no significant difference in the extent of resistance shown against bacteria, fungi or TMV by each independent transgenic line, with single or multiple gene copies (data not shown).

To investigate the ability of transgenic plants to resist bacterial infection, detached leaves from 4–6 weeks old soil grown transgenic and control (non-transgenic and GUS) plants were placed in Petri dishes with wet filter paper. Leaves were wounded and inoculated with 10 μl (~5 × 10^5 cfu) of an *E. carotovora* suspension. After 6 days, the extent of decay was much higher in the control plants as compared to the transgenic plants (Fig. 5A). The transgenic plants expressing 11R (line#16) were more resistant to *Erwinia* than plants expressing 10R (line#6) (Fig. 5A). Infected leaf in this case remained green and showed less decay. The experiment was performed 3 times and similar pattern of resistance was observed.

To study the response of 10R- and 11R-expressing tobacco plants to fungal infection, detached leaf fungal assays were performed. Fig. 5C shows the results of the fungal infection assays on control (non-transgenic and transgenic GUS control) and transgenic plants with single copy of the gene (10R#6 and 11R#16). *Botrytis cinerea* [Fig. 5C (a–d)] and *Verticillium* sp. [Fig. 5C (e–h)] were inoculated on the detached leaves. Six to 14 days after infection the leaves from the non-transgenic tobacco (wt) and transgenic control (GUS) were heavily infected by *Botrytis* and *Verticillium* infection. In contrast, infected areas on the transgenic leaves originated from transgenic plants were much smaller and limited to the area around the site of contact with the fungus containing agar plugs (Fig. 5C). Both, 10R- and 11R-expressing plants showed significant resistance.

Transgenic plants expressing indolicidin variants were tested for resistance to tobacco mosaic virus. Lines 10R#6 and 11R#16 were examined. Lesions on transgenic leaves were counted and compared with the controls (non-transgenic plants) (Fig. 5B). A significant reduction in the number of lesions in transgenic lines (For 10R, and 11R) was observed (Fig. 6A). Similar results with reduction in infectivity in transgenic plants samples were seen by DAS-ELISA of leaf extracts from the lesion forming leaves (Fig. 6C). The dot blot hybridization with

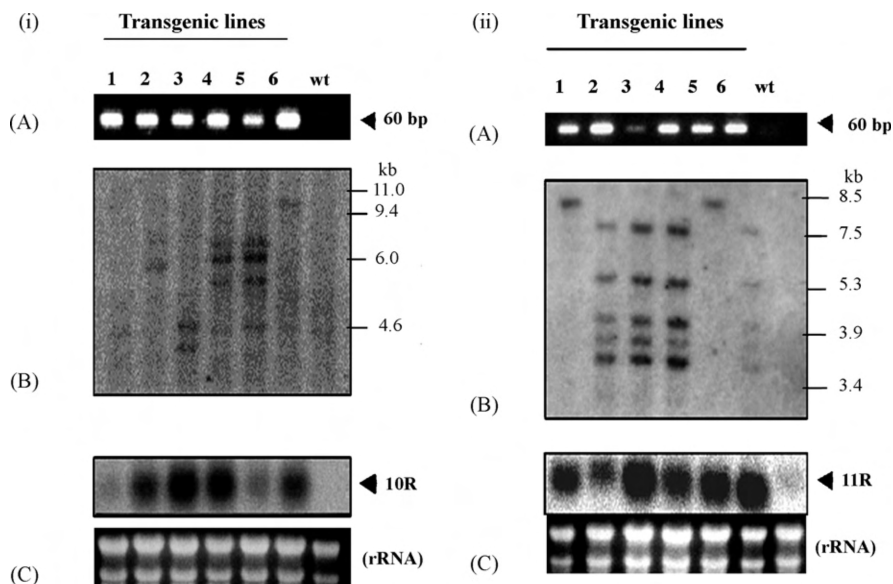

Fig. 4. 10R (i) and 11R (ii) gene integration and expression. (A) The cationic peptide coding sequence was PCR amplified from DNA isolated from control (non-transformed, lane wt) and transgenic (lanes 1–6: transgenic tobacco lines #4, #6, #11, #19, #20 and #21 for 10R and transgenic tobacco lines #1, #7, #12, #13, #16 and #17 for 11R respectively. (B) Southern blot analysis of transgenic plants. Tobacco leaf DNA was digested with *Eco*RI, electrophoresed and hybridized with a ^{32}P-labelled 10R or 11R probe. The number of bands reflects the number of transgene insertions. For lanes description see (A). (C) Northern blot analysis of 10R (i) or 11R (ii) mRNA accumulation in tobacco. Total RNA was prepared from leaves of transgenic plants; RNA samples (30 μg each) were separated by denaturing formaldehyde agarose gel electrophoresis, blotted and hybridized with a ^{32}P-labelled 10R or 11R probe. Ethidium bromide stained ribosomal RNA bands (rRNA) are shown as loading control.

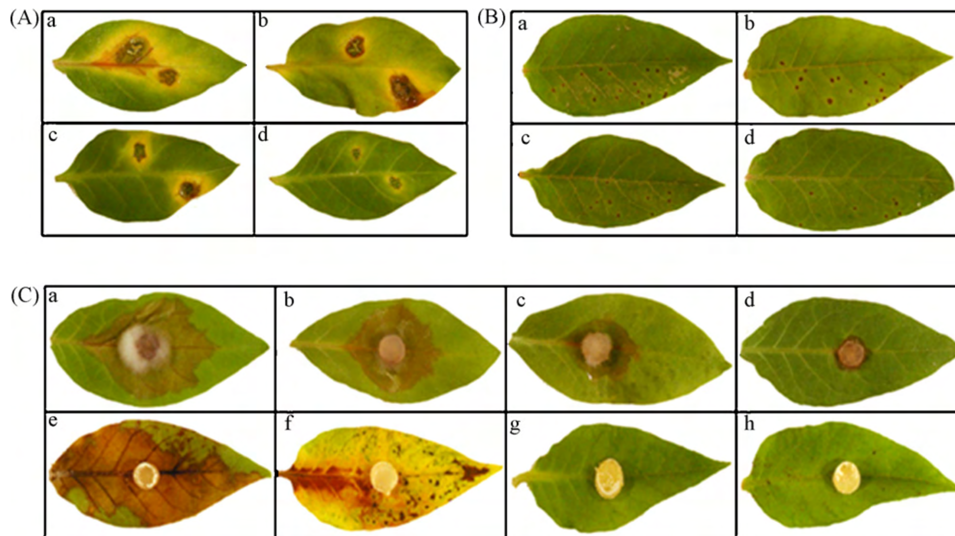

Fig. 5. Resistance of transgenic tobacco expressing indolicidin variants to phytopathogens. Detached leaves from mature nontransgenic tobacco control (a, e), transgenic GUS (b, f) and transgenic tobacco expressing 10R (c, g) and 11R (d, h) were infected with (A) *Erwinia carotovora* (B) TMV (C) *Botrytis cinerea* (a–d) and *Verticillium* sp. (e–h) as described in Section 2. Figure shows representative lines (#6 for 10R and #16 for 11R) with a single copy gene and high expression of the transcript.

TMV movement protein cDNA was performed. The confirmation of the hybridization signal by the ^{32}P radioactivity count for each sample in indolicidin expressing plants was done and indicated the resistance of transgenic plants (Fig. 6B). The results for virus testing in the laboratory conditions show that in the detached leaf assays, the transgenic leaves expressing 10R and 11R are resistance to TMV.

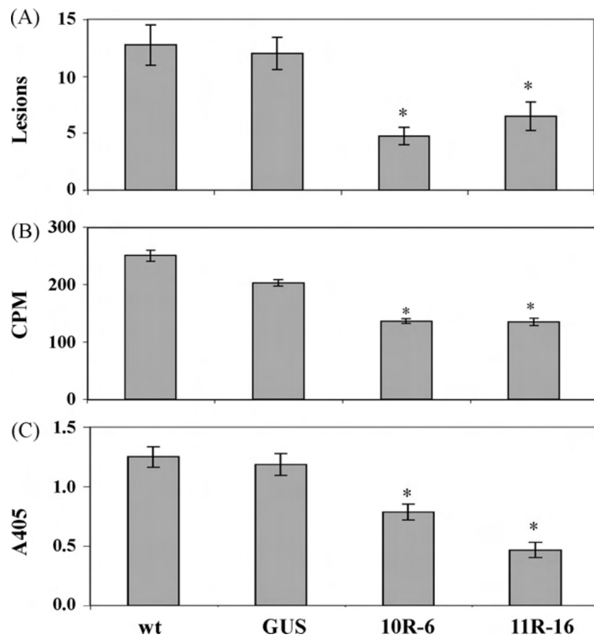

Fig. 6. TMV resistance in transgenic plants. (A) Comparison of lesion numbers in transgenic and control tobacco plants leaves (B) CPM count of the quantitative dot-blot hybridization with TMV movement protein (MP)-cDNA probe and TMV infected 10R and 11R expressing plants. Detached leaves were infected with TMV. Dot-blot was performed as described in Section 2 and extent of hybridization was done by ^{32}P radioactivity count (CPM or count per minute). (C) DAS-ELISA based TMV resistance shown by transgenic tobacco plants expressing indolicidins -10R and 11R. Detached leaves were infected with TMV. ELISA was performed as described in Section 2. A_{405} represents the DAS-ELISA absorbance values at 405 nm. Error bars show S.E.M., $n = 3$. Significant differences between mean values of controls (non-transgenic and GUS) and transgenic tobacco plants are indicated by "*" at $p < 0.05$ (Tukey test).

4. Discussion

The commercial agricultural practices are commonly threatened by frequent bacterial, fungal and viral infections. Pesticides and insecticides have been commonly used to contain phytopathogens; however, their extensive usage has contributed to the chemical contamination of the environment. Attempts have been made to augment plant defenses against microorganisms by genetically engineering plants to express cationic peptides [19,28–31,38]. This approach has proven effective for combating bacterial and fungal diseases in plants. Because the primary target of the cationic peptides is the cell membrane or the nucleic acids and not specific receptor or substrate, these peptides confer their activity against a broad-spectrum of pathogens and there is less probability of resistance arising by variation of its metabolic pathways. There is no report of tapping the antiviral property of these cationic peptides, shown *in vitro* against animal viruses, for providing viral resistance in plants. Therefore, the focus of this study was on antiviral cationic peptides. To confer broad-spectrum resistance against plant pathogens including viruses, indolicidin variants were screened for their *in vitro* antimicrobial activity, expressed in plants by genetic engineering and assessment of disease resistance of the transgenic plants was performed.

*Erwinia carotovor*a was previously shown, to be highly sensitive to cationic peptides [28–30] and similar activity was demonstrated by 10R and 11R. The weak Gram-negative bactericidal activity of 10R as compared to 11R against normally sensitive, LPS-deficient strains of *E. coli* suggested that the low positive charge of the variant may not interact well

with the negative charge of the outer membrane [29,39]. 11R with an increased positive net charge, showed enhanced activity against *E. coli* (data not shown). The minimum inhibitory concentration (MIC) of 11R (10 μg ml^{-1}) for *E.carotovora* were 2–4 times higher than that of other peptides described earlier [28,29]. However, the MIC of 10R was similar to MsrA3 (40 μg ml^{-1}) and MsrA2 (50 μg ml^{-1}) [29].

The *in vitro* efficacy of 10R and 11R against plant pathogenic fungi differed. The antifungal activity of 10R and 11R was cultivar-specific and there was no general trend seen for these peptides against tested fungal strains (data not shown). Indolicidin possess antiviral activity against animal viruses [9,10]. There are no reports describing *in vitro* antiviral activity of an indolicidin against plant viruses. However, the derivatives of indolicidin showed antiviral activity in this study towards TMV. *In vitro* testing of 10R and 11R peptides against TMV on tobacco showed a reduction in viral infection as demonstrated by a reduction in the number of lesions and subsequently by ELISA and dot blot hybridization. This is the first report of antiviral activity of indolicidin peptides against a plant virus. 11R showed *in vitro* anti-TMV activity only at 100 μg of peptide as compared to 10 μg for 10R and thus indicated that the increment in the charges of cationic peptides do not always result in the enhancement of pathogenic activity. 10R showed elevated CPM (Fig. 3B) and high intensity (Fig. 3A) in 100 μg treated TMV sample as compared to 10 μg treated sample. However, ELISA, which is more sensitive assay than dot blot or CPM count, suggested the higher activity of 100 μg 10R against TMV. The difference seen in the dot blot can be due to uneven washing during the hybridization procedure. *In planta* studies showed that tobacco plants expressing 10R and 11R are resistant to 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 [31,38].

Experiments with detached leaves from transgenic tobacco plants demonstrated bactericidal activities when tested with *E. carotovora*—the primary cause of bacterial soft rot in plants. Similar results have been shown earlier with *E. carotovora* and expression of other cationic peptides in plants [28,29,40]. This is in agreement with the strong *in vitro* antibacterial activity seen for the synthetic derivatives of the expressed peptides (Fig. 2).

Plants expressing 10R and 11R showed resistance to *Botrytis* and *Verticillium*. Because the increased resistance was observed only in plants expressing these peptides, the results strongly suggest that the resistance of transgenic tobacco plants is caused by the expression of the 10R and 11R.

In addition to broad-spectrum bacterial and fungal resistance, transgenic plants expressing 10R and 11R are resistant to TMV. A new strategy for engineering virus-resistant plants by transgenic expression of a dominant interfering peptide was shown by Rudolph et al. [25] presented a promising strategy for expressing small peptides in plants. Expression of only a short peptide or artificial peptides minimizes the potential deleterious effects on the plant cells. It is clear from this work that although these antimicrobial peptides are broad-spectrum peptides, their activity does show specificity for certain strains of fungi and bacteria. Similar specificity against other plant viruses is expected.

Detection of the expressed cationic peptides is a challenging task. Small size and high positive charge of these peptides make it difficult to raise antibodies against them. Unsuccessful attempts were made in this work to raise antibodies against these variants (data not shown). Recently, detecting of dermaseptin derivative in plants using antibodies was reported [28] and therefore, it will be interesting to undertake different approach for generating antibodies against indolicidin variants.

Although indolicidin is presumed to act on bacteria and fungi by disrupting membranes [37], its mechanism of action remains to be established. Cationic antimicrobial peptides have shown to form pores in membranes, leading to cell lysis. Direct inactivation of the HIV by indolicidin has been reported [9]. However, electron micrographs showed no change on morphology of TMV even at 100 μg of 10R or 11R treatments (data not showed) indicating other mechanism of TMV inactivation than direct disintegration. Interestingly, indolicidin has also been shown to permeabilize bacteria, without causing cell lysis [37]. Indolicidin bind to calmodulin [41], showing the changes in cytoplasmic permeability and the transport of the peptide across the membrane and mainly reduces synthesis of DNA of the pathogen [42]. Further studies are required to address the specificity and conditions of inhibition of gene expression if any, or any other mechanism of action of indolicidins on plant viruses.

Acknowledgements

This work was supported by an NCE-CBDN and an NCE-AFMnet grant to Santosh Misra.

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