Plant Biotechnology 29, 285–291 (2012)
DOI: 10.5511/plantbiotechnology.12.0412a

Original Paper

Transgenic *Petunia hybrida* expressing a synthetic fungal chitinase gene confers disease tolerance to *Botrytis cinerea*

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Received November 10, 2011; accepted April 12, 2012 (Edited by K. Hiratsuka)

Abstract Disease resistance potential of a synthetic fungal chitinase (*NIC*) gene was evaluated in transgenic *Petunia hybrid* employing *Agrobacterium tumefaciens*-mediated genetic transformation. The *NIC* gene (1271-bp in length) was synthesized to encode the same amino acid sequence (except ^{25}Ser to ^{25}Arg) as that of chitinase 1 gene (*chi1*) from *Rhizopus oligosporus.* As 18% of codons in the *NIC* gene were changed from fungal type (AT-rich) to plant type (GC-rich), the nucleotide sequence of *NIC* had 82% homology with the *chi1* gene. *Petunia hybrida* 'Danty Lady' was transformed with *A. tumefaciens* EHA 101 harboring a binary vector plasmid containing *NIC* (gene of interest) and *nptII* (selection marker) genes. Putative transgenic plants were produced on MS medium containing kanamycin monosulphate as a selective chemical. PCR analysis revealed that the *NIC* and *nptII* genes are integrated into the genome of transgenic plants. Integrations of the transgenes were indicated by Southern blot analysis of genomic DNA from the transgenic plants. Expression of *NIC* gene at the mRNA level was confirmed by reverse-transcription-polymerase chain reaction (RT-PCR). Western blot analysis detected the accumulation of NIC protein in the leaves of transgenic plants. The transgenic plants exhibited enhanced resistance against *Botrytis cinerea* (grey mold) as indicated by inhibition of the fungal growth in detached leaves and by the total protein extract. From these results it could be concluded that the *NIC* gene was successfully integrated into the genome of transgenic petunia plants and produced the NIC protein. Expression of the synthetic chitinase, *NIC gene*, probably, conferred enhance resistance to *B. cinerea* in transgenic petunia.

Key words: *Agrobacterium*-mediated transformation, *Botrytis cinerea*, fungal chitinase, *Petunia hybrida*, synthetic gene.

Since the beginning of agriculture, fungal, bacterial and other pathogens have intimate and highly evolved interactions with cultivated plants, resulting in serious outbreak of diseases. Despite the many grounds of control measures applied, such diseases are still appealing threats to the crops. Agriculture worldwide suffers from production losses due to pathogens and pests. It is estimated that without the application of various crop protection measures, crop losses due to pathogens and pests globally would have reached 40% of the attainable production (Oerke et al. 1999).

Consolidated efforts have resulted in controlling plant diseases to a minimal level mainly by application of effective microbiocidal chemicals and deployment of disease-resistant genes in crop species. However, due to the biologically dynamic nature of plant pathogens, plant disease epidemics still occur regularly. This is mainly because of the emergence of either more virulent strains of the pathogen that overcome the resistance genes or strains that are less sensitive to microbiocides (Fry and Goodwin 1997). Also, the use of some chemicals in disease management has raised the issue of environmental concerns.

Traditional breeding aimed at transferring desired traits into established cultivars often results in loss of desirable combinations of useful genes. Under these circumstances, genetic engineering could be utilized effectively to introduce resistance genes from diverse sources into commercial cultivars without loss of existing, desirable traits.

In this study we used a synthetic gene named as *NIC* (Nakamura Ikuo Chitinase), which encodes for identical amino acid sequence to that of chitinase 1 in *Rhizopus oligosporus* (Yanai et al. 1992) but its nucleotide sequence was designed to adjust to codon usages of plants. The chitinase 1 (*chi1*) protein has been reported to exhibit enhanced resistance against *B. cinerea* in transgenic tobacco (Terakawa et al. 1997). We expect the *NIC* gene will show high chitinolytic activity against the cell walls of *R. oligosporus* as well as those of phytopathogenic fungi.

Chitinases which hydrolyse chitin (homopolymer of 1,4-*p*-linked *N*-acetyl-D glucosamine) have been found in bacteria, plants, insects, humans and fungi (Flach et al. 1992; Collinge et al. 1993; Boot et al. 1995) and thought to function in assimilation of chitin, defense against

This article can be found at http://www.jspcmb.jp/
Published online June 6, 2012

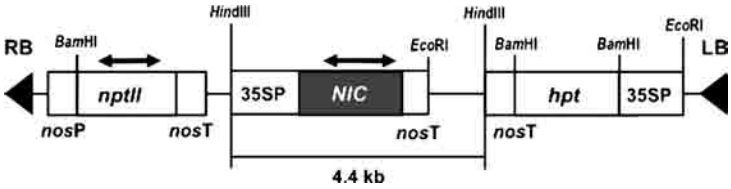

Figure 1. T-DNA region of the binary vector *pEKH1-NIC*. The genes for *NIC* and hygromycin phosphotransferase (*hpt*) are driven by cauliflower mosaic virus 35S promoter (35SP), and the gene for neomycin phosphotransferase (*nptII*) by nopaline synthase gene promoter (*nos*P). The terminators (*nos*T) of the *NIC*, *hpt* and *nptII* genes are also derived from the nopaline synthase (*nos*) gene. Double arrows indicate amplified PCR products for *NIC* and *nptII* genes. LB: left border sequence of the T-DNA, RB: right border sequence of the T-DNA. Recognition sites of restriction enzymes (*Bam*HI, *Hin*dIII, and *Eco*RI) are also indicated. *Hin*dIII-fragment (4.4 kb) of T-DNA was detected by Southern blot analysis.

fungal pathogens and separation of dividing cells. In filamentous fungi, chitinases are thought to be involved in processes requiring cell wall digestion (Gooday 1990), viz. germination of spores, tip growth of hyphae (Bartnicki-Garcia 1973), branching of hyphae, hyphal autolysis and differentiation into spores, as well as in assimilation of chitin and mycoparasitism.

We sought to evaluate the expression and disease resistance potential of *NIC* gene in *Petunia hybrida* as a model plant. *Petunia hybrida* is characterized by both transformability and excellent genetics. Petunias are among the most popular bedding plants in the world because of its versatility, variety, and basic color range, but incidence of pathogens affect badly quality and market value of petunia flowers. The most common disease of *P. hybrida* is grey mold caused by *Botrytis cinerea*. *Botrytis* readily attacks tender succulent tissues, wounded tissues, and senescing plant parts and flowers. A diagnostic feature of this fungus is its ability to produce characteristic brown, fuzzy masses of airborne spores on surfaces of necrotic or senescing tissues.

In this study, we report the development of transgenic *P. hybrida* lines exhibiting enhanced resistance against *B. cinerea* as a result of expression of a synthetic fungal chitinase gene.

Materials and methods

Vector constructs

The *NIC* (1271-bp in length) gene was synthesized to encode the same amino acid sequence (except ^{25}Ser to ^{25}Arg) as that of chitinase 1 gene (*chi1*) in *Rhizopus oligosporus* RCHCHIT1, a zygomycete filamentous fungus (Yanai et al. 1992), although nucleotide sequence of *NIC* gene had 82% homology with the *chi1* gene. The nucleotides at third position of about 18% codons in the *NIC* gene were substituted from fungal type (AT-rich) to plant type (GC-rich), based on codon usage database (http://www.kazusa.or.jp/codon/). The sequence of *NIC* gene used in this experiment was submitted as AB542910 in DNA Data Bank of Japan. A 1.3 kb of *Bam*HI-*Xho*I fragment containing *NIC* gene was inserted between promoter of CaMV 35S transcript and terminator of nopaline synthase gene in pS221s plasmid, a pBI221-like plasmid with spectinomycin resistant gene (Figure 1). The constructed entire plasmid containing chimeric gene (pS221s-*NIC*) was integrated into *Hin*dIII site of pEKH binary Ti vector between kanamycin- and hygromycin-selection marker cassettes (I. Nakamura unpublished). The final constructed plasmid *pEKH-NIC* was transferred from *E. coli* TOP10 to *A. tumefaciens* disarmed strain EHA101 by tri-parental mating (Ditta et al. 1980).

Preparation of inoculum

A. tumefaciens was grown overnight on a rotary shaker (130 rpm) at 28°C in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, pH 7.2) containing 50 mg l^{-1} kanamycin sulphate, 25 mg l^{-1} chloramphenicol, and 100 mg l^{-1} spectinomycin. The bacterial culture was centrifuged for 10 min (3,000×***g***), removed the supernatant and re-suspended the bacterial pellet in hormone-free MS medium, diluted to A_{600}=0.5, and 100 μM acetosyringone (3′–5′-dimethoxy-4′-hydroxyacetophenone; Sigma Aldrich) was added.

Plant material and transformation

Plants of *Petunia hybrida* 'Dainty Lady' cultured under in vitro were used as the experimental material. Regenerated plants were multiplied from the apical and nodal cuttings on hormone-free half-strength MS medium (1/2 MS medium; Murashige and Skoog 1962) in glass bottles (20×100 mm).

For transformation of *Petunia hybrida* by *A. tumefaciens*, leaf explants were incubated in the overnight-grown bacterial inoculum (OD_{600}=0.5) for 5 min, blotted dry with sterilized filter paper to remove excess bacteria, and cultured on MS medium supplemented with 30 g l^{-1} sucrose, 0.25% gellan gum (Gelrite; Kelco, Division of Merck, San Diego, CA), 1.0 mg l^{-1} BAP (benzylaminopurine), 0.1 mg l^{-1} NAA (naphthalene acetic acid) and 100 μM acetosyringone, for 3 days under the dark condition for co-cultivation.

Selection and regeneration

After co-cultivation, the explants were washed with liquid hormone-free MS medium containing 10 mg l^{-1} meropenem (Sumitomo Pharmaceuticals, Osaka, Japan) and transferred on to 0.25% gellan gum-solidified MS medium containing 3% sucrose, 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 50 mg l^{-1} kanamycin monosulphate as a selective antibiotic and 20 mg l^{-1} meropenem as bactericide. The antibiotics were

filter-sterilized and added to the medium after autoclaving. The infected explants were incubated in growth chamber at room temperature for 2 weeks, with weekly subculture to the fresh medium, and then the explants were transferred to 0.25% gellan gum-solidified MS supplemented with 3% sucrose, 1.0 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 100 mg l^{-1} kanamycin and 20 mg l^{-1} meropenem for callus/shoot formation. Explants were subcultured at 2-week interval on this medium. Approximately, 2 months after co-cultivation, adventitious shoots of 1–2 cm long were excised from the discs and cultured on 0.3% gellan gum-solidified MS supplemented with 100 mg l^{-1} kanamycin and 15 mg l^{-1} meropenem for rooting of putative transformants.

PCR analysis

Polymerase chain reactions (PCR) were carried out to screen transformants for the *NIC* and *nptII* genes integration. Genomic DNAs from kanamycin-resistant and control petunia plants, 3–4 weeks old, were extracted using a modified CTAB method (Rogers and Bendich 1988). Amplification was carried out in a thermal cycler (PTC-200 Peltier Thermal Cycler)) in 30 cycles of 94°C for 30 s (*NIC*) and 60 s (*nptII*), 59°C for 30 s (*NIC*) and 60 s (*nptII*), and 72°C for 60 s (*NIC*) and 90 s (*nptII*). The sequences of the PCR primers were as follow:

NIC1, 5′-GGT CGA TGC CGT CCT CCT GTC CTT-3′;
NIC2, 5′-CGC CTT GGT GGT GGT CTT GAT GGT3′;
nptII1, 5′-GAG GCT ATT CGG CTA TGA CTG-3′;
nptII2, 5′-ATC GGG AGC GGC GAT ACC GTA-3′.

Southern blot hybridization

For Southern blot hybridization (Southern 1970), *Hin*dIII-digested DNA samples from kanamycin-resistant and non-transformed control plants were separated on a 0.8% (w/v) agarose gel, blotted to a positively charged nylon membrane (Hybond-N^+; Amersham Pharmacia Biotech, Amersham, UK) and hybridized with a digoxigenin (DIG)-labeled probe of the *NIC* gene. The probe DNA fragments were labeled by PCR using DIG-dUTP, following the supplier's instructions (Boehringer Ingelheim, Ingelheim am Rhein, Germany). Hybridization with the DIG-labeled probes was performed for 16 h at 41°C (McCabe et al. 1997). Hybridization patterns were detected with the chemiluminescent substrate CDP-Star (Roche Molecular Biochemicals, Mannheim, Germany) and anti-digoxigenin-AP (Roche Molecular Biochemicals, UK). The hybridized blot was exposed to Hyperfilm TM-MP X-ray film (Amersham Pharmacia Biotech) for 20 min at room temperature.

Total RNA extraction and reverse transcription (RT)-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA from leaves of in vitro transgenic and non-transformed petunia plants was extracted using an acid phenol–guanidine isothiocyanate–chloroform method (Sambrook and Russell 2001) to detect the expression of the *NIC* gene at the mRNA (mRNA) level. The extracted total RNA was re-suspended in 30 μl DEPC-treated water and quantified with a spectrophotometer (BioMate^{TM} Thermo Spectronic, Rochester, NY).

RNase-free DNase (Qiagen) was used to ensure complete removal of DNA from RNA samples prior to RT-PCR. First strand cDNAs were synthesized from 1 μg of total RNA using *NIC* gene specific primer and a SuperScript Transcriptase III kit (Life Technologies, Carlsbad, CA). The reaction was incubated at 55°C for 50 min and stopped the reaction at 70°C for 15 min. The cDNA was denatured prior to the PCR analysis at 94°C for 2 min. PCR amplification was then performed in 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min.

SDS-PAGE and western blot analysis

To evaluate the expression of the integrated *NIC* in transgenic plants, total proteins were extracted from fresh leaves of transgenic and non-transformed petunia plants. The samples were grinded in liquid nitrogen and homogenized with the protein extraction buffer [(62.5 mM Tris-HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol)] supplemented with 0.2% β-mercaptoethanol. The samples were centrifuged (20,000×***g***) for 10 min at 4°C and the total proteins in the supernatant were denatured by boiling for 3 min followed by chilling on ice for 2 min. The denatured proteins were fractionated on a 12.5% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (Laemmli 1970). Molecular masses of the proteins were determined on the SDS-PAGE gel stained with Coomassie Brilliant Blue by comparison to PageRuler Unstained Protein Ladder in the range of 10.0–200 kDa (Fermentas Life Sciences). The separated proteins were electroblotted using a Bio-Rad electrophoresis system per the manufacturer's instructions (Bio-Rad, Hercules, CA) onto a polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences) for 30 min at room temperature. Immunodetection of transgenically produced NIC protein was carried out on the PVDF membrane incubated at room temperature with a polyclonal antibody prepared against the purified NIC protein. The PVDF membrane was blocked for 1 h in 5% BSA in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl). The blot was incubated for 1 h against primary antibody in TBS (at a dilution of 1 : 1000, v/v) containing 5% bovine serum albumin (BSA), subsequently with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) as a secondary antibody (1 : 100,000, v/v) (Amersham Biosciences, USA) for 1 h using enhanced chemiluminescence (ECL) western blotting detection kit (Amersham Bioscience).

Fungal resistance assay using detached leaves of NIC-transgenic plants

We chose two transgenic lines (Figure 4C lanes 4, 5) showing high protein expression to assay resistance against *Botrytis cinerea* as a model system. For testing disease resistance potential of *NIC* in the detached leaves, spores suspension (inoculum) was prepared from 2-weeks old culture of *B. cinerea* strain 40 grown on PDA in plastic Petri dishes (20×90 mm).

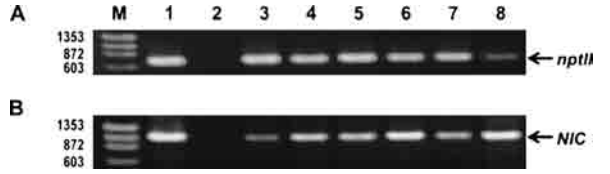

Figure 2. PCR analysis of genomic DNA from the transgenic and non-transformed *P. hybrida* 'Dainty Lady' obtained after infection with *A. tumefaciens* harboring *NIC* and *nptII* genes. (A) Amplification of the selection marker gene (*nptII*) (0.8 kb). (B) Amplification of *NIC* gene (1.2 kb). Lanes M: Size marker (*φX174*/*Hae*III digests), 1: positive control (plasmid DNA), 2: non-transformed, 3–8: independent transgenic petunia plants. Arrows indicate the amplified fragments of *NIC* and *nptII* transgenes.

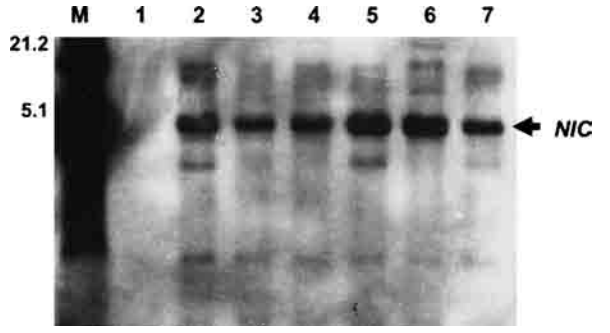

Figure 3. Southern blot analysis of genomic DNA from transgenic and non-transformed *P. hybrida* 'Dainty Lady' obtained after infection with *A. tumefaciens* harboring *NIC* and *nptII* genes. *Hin*dIII-digested genomic DNA from transgenic and non-transformed petunia was hybridized with a DIG-labeled probe of the *NIC* gene. Lanes M: DIG-labeled molecular weight marker III, 1: non-transformed, 2–7: independent transgenic lines.

The cultures were flooded with 5 ml of sterilized distilled water and used a Drigalski spatula to release the spores gently from mycelia. Spores from all the Petri dishes were collected with a sterile Pasteur pipette, mixed, counted with a haemocytometer and their concentration was adjusted to 1×10^6 spores per ml using sterilized distilled water. Eighteen young mature leaves from 6–7 week-old the three transgenic lines Tr2, Tr4, and Tr5 (Figure 4B lanes 2, 4, 5) and the same from control plants (Nt1, Nt2, Nt3) were harvested and placed on wet filter paper in Petri dishes, wounded in the middle on both sides of midrib, and inoculated with the inoculum (1×10^6 spores/ml) of *B. cinerea* strain 40 (Figure 5A, B). The inoculated leaves were incubated at 24°C and 100% relative humidity under a 16/8-h (light/dark) photoregime for a week. Pictures were taken a week after inoculation (Figure 5A, B).

Effect of extract from NIC-transgenic petunia on growth of B. cinerea

Antifungal effect of the NIC protein in the two transgenic lines Tr4 and Tr5 (Figure 4B lanes 4, 5) was evaluated as described (Broekaert et al. 1997; Cammue et al. 1992) with slight modification. Briefly, proteins were extracted from the leaves of transgenic and non-transformed *P. hybrida* to evaluate their antifungal effect on growth of fungal hyphae. Fifty microlitre

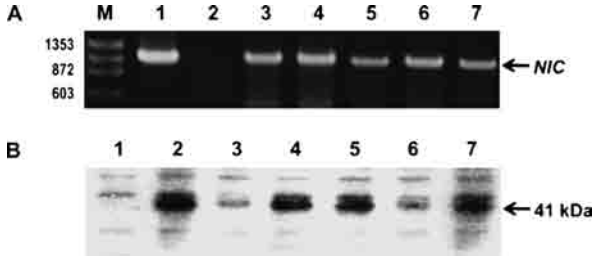

Figure 4. RT-PCR and western blot analyses of transgenic and non-transformed *P. hybrida* 'Dainty Lady' obtained after infection with *A. tumefaciens* harboring *NIC* and *nptII* genes (A) Reverse transcription (RT)-PCR was performed to confirm the expression of *NIC* gene at the mRNA level. PCR amplification of cDNAs from transgenic plants (lanes 3–7) and the plasmid control template (lane 1) yielded the expected product of 1.2 kb, whereas the non-transformed plant did not produce the product (lane 2). (B) Western blot analysis for the expression of *NIC* protein in transgenic petunia plants. Protein extracts of leaves from in vitro transgenic and non-transformed plants were fractionated on a 12.5% polyacrylamide gel and subjected to immunoblot analysis using a rabbit polyclonal antiserum for NIC protein. Lanes 1: non-transformed, 2–7: transgenic plants with varying accumulation level of NIC protein.

(50 μg) of the total protein extract and 50 μl of spore suspension (1×10^6 spores/ml) of *B. cinerea* were mixed with 100 μl of potato dextrose broth (PDB) in a well (18×20 mm) of a microtitre plate (Falcon Multiwell™) and incubated at 24°C on orbital shaker (100 rpm) for 48 h. The culture was then stained with 50 μl of tryphan blue solution (0.02 g of tryphan blue, 10 g phenol, 10 ml lactic acid, 10 ml glycerol, and 10 ml distilled water), incubated at room temperature for 5–10 min and measured the absorbance at 595 nm with a spectrophotometer (BioMate™ Thermo Spectronic, Rochester, NY). Spores germination and growth of hyphae were estimated by the increased absorbance (AU) after 48 h of inoculation.

Results and discussion

In this study, *Agrobacterium*-mediated transformation was employed to transform petunia with a modified fungal chitinase gene (*NIC*) using *nptII* as a selection marker to confer enhanced tolerance to *B. cinerea* (gray mold). Although shoot regeneration occurred at several positions of the explant (leaf disc), 2–3 well-grown shoots were transferred to rooting medium, and finally one well-rooted shoot was selected from each explant for further analysis, representing an independent putative transgenic line.

Molecular analyses of transgenic plants

PCR analysis of the kanamycin-resistant lines confirmed integration of the transgenes, *NIC* and *nptII*. All the clones showed amplified fragments of approximately 0.8 kb for *nptII* (Figure 2A, lanes 3–8) and 1.2 kb for *NIC* (Figure 2B, lanes 3–8) genes, suggesting that the T-DNA of the binary plasmid vector was successfully integrated into the genome of transgenic plants. No amplified band

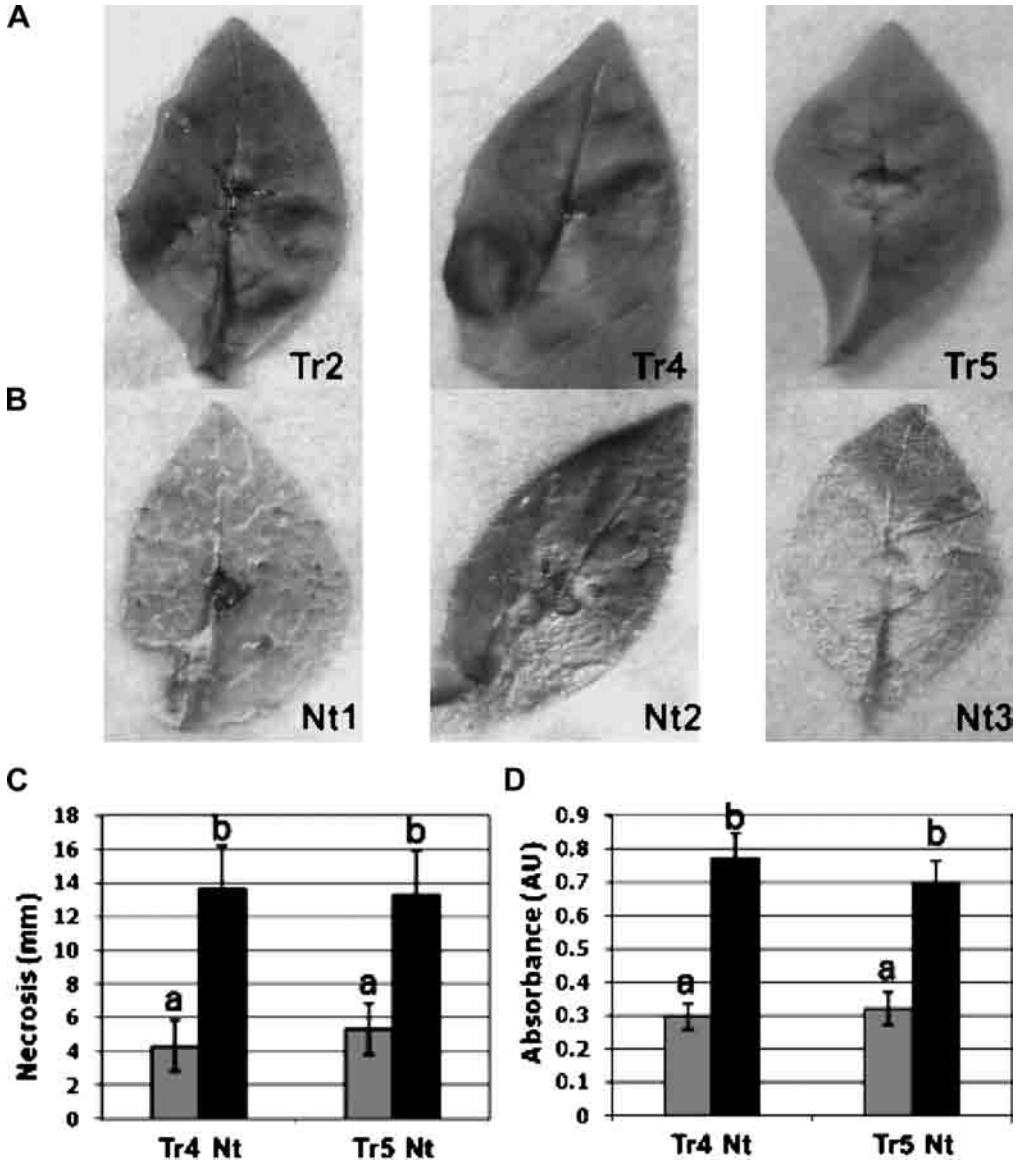

Figure 5. Antifungal resistance assay of the *NIC*-transgenic petunia plants. Detached leaves from mature (A) transgenic lines (Tr2, Tr4, Tr5, and (B) non-transformed lines (Nt1, Nt2, Nt3) were challenged with the spore suspension (1×10^6 spores/ml) (20 μl each) of *B. cinerea*. After inoculation, the leaves were incubated at 25°C under 16 h light/8 h dark conditions and high humidity for a week. Pictures were taken 7 days after inoculation. After a week, the leaves from the non-transformed plant decomposed, possibly, because of the fungal infection, whereas the leaves from the transgenic plants expressing *NIC* were still green with small necrotic spots only at the sites of inoculation. (C) Area (mm^2) of necrotic lesion developed on detached leaves of transgenic (Tr4, Tr5) and non-transformed (Nt) petunia plants after inoculation with the spores of *B. cinerea*. (D) Growth of *B. cinerea* in crude protein extracts from transgenic (Tr4, Tr5) and non-transformed (Nt) *P. hybrida* leaves. Absorbance of the reaction mixture (crude protein extract+spore suspension+potato dextrose broth) stained with trypan blue was measured at 595 nm. Bars represent means and standard errors and those with different lower case letters are significantly different at ($p>0.05$) by least significant difference (LSD) test. The results represent the average and standard deviation of three experiments.

was shown in PCR products from genomic DNA of non-transformed control plants.

Southern blot analysis showed the same size bands (4.4 kb, Figure 3) in lanes containing *Hin*dIII-digested genomic DNA of *NIC*-transgenic plants. The results confirmed integration of transgenes into the genome of all the kanamycin-resistant lines (Figure 3, lanes 2–7). No transgene insertion was detected in non-transformed control (Figure 3, lane 1). Copy number of the transgenes could not be determined in this study.

RT-PCR was carried out to confirm the expression of *NIC* gene at the mRNA level (Figure 4A). Amplified products were detected in all the transgenic lines (Figure 4A, lanes 3–7) but not detected in the control plant (Figure 4A, lane 2).

SDS-PAGE was carried out to fractionate total protein extracts of leaves from the PCR-positive transgenic lines and non-transformed control plant. To further evaluate the expression of the integrated *NIC* gene in genomic DNA of transgenic plants, the protein extracts were

subjected to western blotting using antiserum raised in rabbit against synthetic peptides of NIC protein (Figure 4B). In western blot analysis of protein extracts from the *NIC*-transgenic and non-transformed control petunia plants detected a 41-kDa band corresponding to NIC with varying intensity in the total protein extracts of transgenic plants (Figure 4B, lanes 2–7). Nonetheless, the western blot also detected a few bands of low intensity in transgenic as well as non-transformed plant (Figure 4B). Appearance of these bands could be attributed to the endogenous chitinases cross-reacting non-specifically with the *NIC* polyclonal antibody.

It has been reported that accumulation levels of the protein may vary widely among the individual transformants (Fagard and Vaucheret 2000). Meyer et al. (1995) reported that a position effect in the genome may result in the differences in the levels of transgene expression.

Resistance of transgenic plants expressing NIC gene against B. cinerea

Fungal resistance of transgenic plants expressing the *NIC* gene was evaluated against *B. cinerea* (grey mold), one of the major pathogenic fungi, which causes severe damage to the leaves and flowers of petunia.

Disease resistance assay of mature leaves detached from transgenic and control petunia was performed against *B. cinerea*. Necrotic lesions were developed on the control leaves that expanded with time as more tissue was damaged by the pathogen. Area of necrotic spots on the leaves was measured a week after inoculation (Figure 5C). After 6 days of incubation, most of the leaf area from the control plant turned brown and decomposed because of the fungal infection (Figure 5B), whereas the leaves from the transgenic plants expressing *NIC* were still green with small necrotic lesions only at the sites of inoculation (Figure 5A). Microscopic studies of the infected area of the leaf from the transgenic and control plants showed that NIC protein restricted growth and multiplication of the fungal hyphae. A network of hyphae could be seen in the dead tissue of control leaf infected with *B. cinerea* a week after inoculation (data not shown).

This enhanced resistance against *B. cinerea* by overexpression of the *NIC* gene supports the previous report describing antifungal activity of *Chi 1* from *R. oligosporus* in transgenic tobacco (Terakawa et al. 1997). This is also in parallel to our previous study where enhanced resistance potential of *ChiC* (from *Streptomyces griseus*) against *Alternaria solani* in transgenic potato plants was reported (Khan et al. 2008).

Effect of crude leaf extract on the growth of B. cinerea

The inhibitory effect of leaf crude protein extracts from *NIC*-transformed and control petunia plants on growth of *B. cinerea* was evaluated. Quantitative measurement of hyphae growth in presence of protein extracts showed that growth of hyphae was restricted in transgenic plants expressing *NIC* gene compared to that in the presence of protein extracts from the control plant (Figure 5D). This further confirms the antifungal effect of NIC protein.

The mode of action of *NIC* might be attributed to its restriction of hyphal growth and cell wall degradation supporting the results of Yanai et al. (1992) where they isolated and characterized chitinase 1 and chitinase 2 from *Rhizopus oligosporus*. The higher resistant effect of the *NIC*-transgenic petunia plants indicated that over-expression of an introduced chitinase gene possibly accelerated the expressions of other related chitinolytic enzyme (such as glucanase) genes, as observed in many other herbaceous and woody plants (reviewed in Lorito and Scala 1999; Harman et al. 2004). It seemed that the transgenic plants responded to the pathogen attack more efficiently than the control, possibly, because of the over-expression of abundant PR proteins in the former ones.

These preliminary pathogenicity tests demonstrated antifungal resistance of the *NIC*-transgenic plants against *B. cinerea*. However, further research is needed to test the resistance of the *NIC*-transgenic plants against other pathogenic fungi and bacteria.

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