Expression of an antimicrobial peptide (MSI-99) confers enhanced resistance to *Aspergillus niger* in transgenic potato

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MSI-99, a synthetic substitution analogue of antimicrobial peptide magainin, was used to impart enhanced resistance against fungal pathogen, *Aspergillus niger* in transgenic potato cultivars, Kufri Jyothi and Kufri Bahar. Internodal stem segments were transformed with *Agrobacterium tumefaciens* strain EHA105. Integration of the transgene, MSI-99 in the transgenic plants of both the cultivars was confirmed by PCR and PCR-Southern analysis, exhibiting 180 bp fragment and hybridization with labelled probe pSAN168. Bioassay studies using detached leaves of transgenics showed the enhanced resistance against *A. niger*.

**Keywords:** antimicrobial peptides, *Aspergillus niger*, MSI-99, transgenic potato

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**Introduction**

Phytopathogens have been responsible for huge amount of crop losses, which can be minimized by effective and efficient technological manipulations to achieve disease resistant in plants. Genetic engineering is such an alternative-way, which has been employed to develop disease resistant crops. Further, incorporation of antimicrobial peptides has successfully been used to enhance broad-spectrum fungal and bacterial disease resistance in crop plants.

The antimicrobial peptides are small, cationic and pore-forming peptides, having a wide occurrence among amphibians, molluscs, higher vertebrates and even plants. The wide spectrum of activity of these peptides at a concentration relatively non-toxic to eukaryotic cells and rapid mode of synthesis, either constitutively or upon infection with a minimum input in terms of energy and biomass, makes them attractive choices. Magainin is one of the earliest reported antimicrobial peptides. It is a 23-aminoacid-long, α-helical peptide, effective against Gram-positive and Gram-negative bacteria, fungi and protozoa. Recently, a magainin analogue named Myp30 has been used to enhance the disease resistance in tobacco. Similarly, another substitution analogue MSI-99, expressed in tobacco chloroplasts, conferred both *in vitro* and *in planta* resistance to phytopathogenic bacteria and fungi.

Potato is the fourth most important food crop after rice, wheat and maize, but its production has been limited mainly due to diseases and insect pests causing considerable losses. In the present communication, authors have demonstrated the transfer of MSI-99 to Indian potato cultivars Kufri Jyothi and Kufri Bahar, resulting in enhanced resistance to *Aspergillus niger*. This fungus is known to grow on many irradiated food materials including potato and is considered as a major cause of rot during storage.

**Materials and Methods**

**Establishment and Maintenance of Potato Shoot Cultures**

Sprouts from potato tubers were detached, washed in running tap water and then twice in 70% ethanol. They were then surface sterilized with 0.1% mercuric chloride for 5 min and repeatedly washed in sterile water to remove the traces of mercuric chloride. Meristematic tips measuring 0.5 to 1.0 cm from these surface sterilized sprouts were excised and used as explants. The explants were cultured on MS medium for establishing the shoot cultures. The pH of the medium was adjusted to 5.8 before autoclaving and the medium was gelled with 0.2% phytagel (Sigma). For the formation of plantlets, all the cultures were
kept in a growth room at 25±2°C, under 15 µE m⁻² s⁻¹ light intensity in a 10/14 h day/night cycle and with 50-60% relative humidity. The cultured sprouts developed into plantlets in about 4 weeks time. Nodes from these plantlets were cultured on MS medium supplemented with 0.29 µM GA₃, 0.54 µM NAA and 0.1% activated charcoal for raising the plants. Internodal segments from these plants were subjected to Agrobacterium-mediated transformation.

Agrobacterium-mediated Transformation

The recombinant binary plasmid pMSI168 (Agrobacterium tumefaciens strain EHA105)¹², containing MSI-99 gene driven by UBQ3 promoter from Arabidopsis and pea vicilin secretory signal to target the peptide into extra cellular space, was used for transformation¹³. In each experiment, 200 internodal segments from in vitro grown plants of Kufri Jyoti and Kufri Bahar cultivars of potato were exposed to Agrobacterium as described earlier¹⁴. The internodal segments (1-2 cm in length) were precultured on MS basal medium for 48 h and were infected with Agrobacterium suspension (0.5 OD) for 30 min on a shaker. Four hundred explants comprising both the varieties were then blotted dry on sterile Whatman no.1 filter paper and were transferred to MS basal medium for co-cultivation for 48 h. Following co-cultivation, the explants were cultured on regeneration media, i.e. MS medium supplemented with trans-zeatin (13.68 µM), IAA (0.57 µM), GA₃ (14.43 µM) containing cefotaxime (400 mg L⁻¹) and kanamycin (50 mg L⁻¹), for regeneration of transgenic shoots and inhibition of Agrobacterium growth. The regenerated shoots were excised and subcultured on MS medium supplemented with NAA (0.54 µM), GA₃ (0.29 µM) and kanamycin (100 mg L⁻¹) for rooting and complete plantlet formation.

Analysis of Potato Transgenics by Polymerase Chain Reaction

Transgenic nature of the plants was confirmed by PCR using DNA from 1-month-old in vitro grown transgenic and control plants. DNA was isolated by CTAB method¹⁵. The upstream primer used for PCR was specific to the secretory signal sequence (P168), while the 3’ end primer was specific to the 5’ region of the nos terminator (P nos). The primer sequence are, P168: 5’-GCCTTCTTGGCATCAGTTTGCCTG-3’; and P nos: 5’-ATCGCAAGACCGGCCAACAGG-3’. 50 ng of genomic DNA was used for PCR analysis in a reaction volume of 50 µL, containing 1X Taq polymerase buffer, 200 µM each dNTP, 1 µM each primer and 1 unit of Taq polymerase (Bangalore Genie, India). The PCR profile used consisted of the following: initial melting at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min with a final extension at 72°C for 10 min. The PCR products were analyzed on 2% agarose gel using 1X TAE buffer.

Southern Analysis of PCR Products

After gel separation, the PCR products were blotted onto nylon membranes (Hybond N+; Amersham-Pharmacia). The 500 bp EcoRI fragment from pSAN168 containing the MSI-99 gene and nos terminator was radioactively labeled with α-[³²P]dCTP, using the random primer labeling kit from Board of Radiation and Isotope Technology (India) according to the manufacturer’s instructions, and used for hybridization. The blotting and subsequent hybridization were carried out as per standard protocol¹⁶.

Bioassay of Potato Transgenics for Resistance to A. niger

Cultures of A. niger was obtained from Agarkar Research Institute, Pune, India and maintained on PDA slants. The third leaf from the top of transformed potato plants as well as untransformed control plants, was excised, surface sterilized with 70% ethanol and placed upside down on moist sterile Whatmann No. 3 paper in petriplates. The leaves were injured with a fine needle at the centre on two sides of the mid rib and were inoculated with 10 µL of spore suspension containing 10⁷ spores/mL. Five leaves from each transgenic line were taken for bioassay. The plates were sealed with Parafilm and incubated at 30°C. The observations on disease were recorded after 3 d and average lesion size was calculated. The experiment was repeated thrice and pooled data was analyzed. The average lesion diameter of individual transgenic lines was compared to that of untransformed control plants to determine the level of resistance.

Results and Discussion

Internodal stem segments cocultivated with Agrobacterium harboring pMSI168 plasmid showed the emergence of shoots on shoot induction media after 3 to 4 weeks in 10 to 15 per cent of the explants. When 50 developing shoots of each variety were excised and transferred to fresh medium of the same
composition, they grew vigorously in the presence of kanamycin (50 mg L⁻¹). Upon transfer to medium containing higher concentration of kanamycin (100 mg L⁻¹), about 90% of the shoots (~45) developed further with profuse rooting. Three independently transformed lines of each variety were selected randomly from the rooted plantlets for PCR and Southern analysis.

The PCR analysis was carried out with six representative samples, three each from Kufri Jyoti and Kufri Bahar, and 180 bp fragment was amplified in all the six transgenics (Fig. 1), whereas these transgene specific bands were absent in the untransformed control plants. The Southern blotting of the PCR products and its hybridization with radioactively labeled gene for MSI-99 along with the nos terminator further confirmed the identity of MSI-99 in the transgenic plants, as only the PCR amplified products from the transgenic plants hybridized with the probe (Fig. 2).

Pathogenicity test on transgenics, performed to study the extent of resistance in transgenic potato plants to A. niger, showed that various transgenic plants produced different types of lesions ranging from 0.5 to 5 mm in diam. However, two transgenic plants—J-2-168 of Kufri Jyoti and B-5-168 of Kufri Bahar showed respectively average lesion diam of 0.8 and 1.2 mm, which were 19 and 28% of that of the untransformed control plants (4.2 mm; Figs 3 & 4).

The main objective of this study was to incorporate fungal disease resistance trait into Indian cultivars of potato using MSI-99, a synthetic analogue of antimicrobial peptide (AMP) magainin. Different AMPs, like Myp30, MSI-99, tachyplesin, cecropin and attacin, have been used in transgenic plants for enhanced disease resistance⁷⁻¹⁹. Although these different AMPs were tested in a variety of crops with different pathogens, the plants expressing magainin type AMPs showed less severe disease symptoms compared to the controls, indicating that these AMPs can be employed for imparting enhanced disease resistance in a variety of crops¹⁷⁻⁸. Chakrabarti et al¹⁰ reported the transformation of tobacco and banana plants with MSI-99 that showed enhanced resistance against Sclerotinia sclerotiorum, Alternaria alternata and Bortrytis cinerea in case of tobacco and against Fusarium oxysporum f.sp. cubense and Mycosphaerella musicola in case of banana. Further, Alan et al¹⁰ showed enhanced resistance in tomato against Pseudomonas syringae pv. tomato, a bacterial speck pathogen, by expressing MSI-99. Antimicrobial peptide, snakin-2 isolated from potato was used to study the inhibitory activity against different fungal pathogens including Aspergillus molds.²⁰ De Grey et al²⁰ reported 95% reduction in germination of A. flavus conidia in vitro in response to the expression of MSI-99 in tobacco chloroplast. In the present study,
transgenic potato plants expressing MSI-99 showed enhanced resistance to A. niger and a maximum reduction of lesion size by 81% was observed in J-2-168.

For significant enhancement of resistance, continuous and high level expression of MSI-99 was suggested to be desirable. Secondly, the peptide should be secreted into extracellular spaces so that phytopathogens were inhibited before they invade the host cell. In the present study, pea vicilin secretory sequence was employed to target the peptide into extracellular space. The transgene derived AMFs would also be expected to be highly labile and degraded rapidly after ingestion by higher eukaryotes or in the soil environment, leaving no toxic residues. Barrell and Conner (personal communication) expressed chimeric magainin gene in potato which conferred improved resistance to the phytopathogen, Erwinia carotovora. They suggested that the production of magainin type AMPs in potato tissues might not pose a risk to human or animal health upon ingestion. These peptides do not have toxicity to plant tissues, but kill the microbes faster than their doubling time. Hence, the chances of emerging resistance might not arise. Antimicrobial peptide MSI-99, a synthetic analogue of magainin, was expressed in chloroplasts of tobacco and was found to have no adverse effect on plant growth and development. Similarly, MSI-99 was expressed in transgenic banana plants without any adverse effect on the growth of these plants. In the present study, transgenic potato plants also grew normally in the green house and did not show any adverse effect on their growth (data not shown).

The present studies with transgenic potato plants expressing MSI-99 showed enhanced resistance to fungal pathogen A. niger, which could be used to impart much required resistance in potato against this fungus for it infects potato tubers during post irrigation storage. Thus, the acquired resistance would be beneficial. Further studies are underway to test the resistance of these transgenic potato plants to other phytopathogens.

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References