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Cloning, Expression and Development of Transgenic Tobacco using *ChiA* gene from Native Isolate of *Serratia marcescens* 141

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ABSTRACT

Serratia marcescens is one of the most effective Gram-negative bacteria for degradation of chitin 1,4-β–glucosamine, which is the major structural component of the exoskeleton of insects and crustaceans and also occurs in the cell wall of several fungi. Chitinases are of biotechnological importance because nature uses these enzymes as a plant biocontrol agent against fungal elicitors. Chitinases hydrolyse the β-1-4 linkage in chitin to soluble oligosaccharides, mainly chitobiose, which is hydrolyzed to GlcNAc by chitinase. Different isolates of *S. marcescens* obtained from soils of Western Ghats were screened for their chitinolytic activity from total DNA which was isolated from the most efficient strain. A 1691-bp amplicons was obtained after PCR amplification with *Chi*A-specific primers. The amplicon was cloned into pTZ57R/T vector. When sequenced, it showed 99% homology with reported sequences both at nucleotide and translated amino acid levels. The *Chi*A gene was expressed in *Escherichia coli* BL21 by subcloning into prokaryotic expression vector pET28a (+) and in tobacco by subcloning into plant transformation vector pHS100.

Keywords: chitin, chitobiose, GlcNAc- N acetyl glucose amine, 1,4-β-glucosamine
Abbreviations: IPTG, isopropyl β-thio-*O*-galactopyramoside; **PCR**, polymerase chain reaction; **SDS-PAGE**, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

INTRODUCTION

Chitin, a linear β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) polysaccharide, is a main structural component of the fungal cell wall and the exoskeletons of invertebrates, such as insects and crustaceans (Wang and Hwang 2000). It is one of the most abundant naturally occurring polysaccharides and has attracted tremendous attention in the fields of agriculture, pharmacology and biotechnology (Antranikian *et al*. 2005; Muzzarelli *et al*. 2005). Chitin can be hydrolysed by an enzyme chitinases. Chitinases catalyses the conversion of chitin and it is produced by a wide range of organisms, including bacteria (Kitamura and Kamei 2003), plants (Masuda *et al*. 2001) and fungi (Ike *et al*. 2006).

Chitinases are a class of hydrolytic enzymes that hydrolyses the β-1,4-linkages in chitin, by either an endolytic or exolytic mechanism. The chitinases so far sequenced are classified into two different families based on the amino acid sequence similarity of their catalytic domains. These form families 18 and 19 in the family classification system of glycoside hydrolases (Henrissat 1991). Family 18 contains chitinases from bacteria, fungi, viruses, animals and some plant chitinases.

Several genera of bacteria including *Serratia* (Watanabe *et al*. 1997; Suzuki *et al*. 2002), *Enterobacter* (Chernin *et al*. 1995), and *Aeromonas* (Wang *et al*. 2003), produce high levels of chitinolytic enzymes. *Serratia marcescens* is one of the most efficient chitin-degrading agents, and many chitinases have been reported from several strains of this species (Horn *et al*. 2006), such as *ChiA*, *ChiB*, and *ChiC1*. Gram-negative soil bacterium *S. marcescens* produces three chitinases (A, B, C), a hexosaminidase, and a chitin binding protein (CBP21) lacking chitinase activity (Suzuki 2002; Vaaje-Kolstad *et al*. 2005).

Genetic engineering offers the potential to improve elite cultivars for individual traits such as disease resistance. Resistance to fungal pathogens is highly desirable. Chitinases from the chitinolytic bacteria such as *S. marcescens* are endolytic enzymes that solubilize chitin more rapidly than the exolytic enzymes and could therefore, be more efficient in controlling fungal diseases of crop plants (Monreal and Reese 1969).

Chitinases from bacteria have been used in suppressing fungal phytopathogens such as *Rhizoctonia solani* and *Aspergillus parasiticus* (Gomes *et al*. 2001; Reyes-Ramírez *et al*. 2004). Two chitinases were purified from *Serratia plymuthica* HRO-C48 (Frankowski *et al*. 2001), both of them showed inhibitory effects on the growth of the plant pathogenic fungus *Botrytis cinerea*. Antifungal chitinases have also been characterized from other sources as well. A 35 kDa chitinase from barley was found to be antifungal against *Botrytis cinerea*, *Pestalotia theae*, *Bipolaris oryzae*, *Alternaria* sp., *Curvularia lunata* and *Rhizoctonia solani* (Kirubakaran and Sakthivel 2007). A chitinase of *Ficus pumila* L. var. awkeotsang (Makino) showed inhibitory effects on spore germination of *Colletotrichum gloeosporioides*, which causes mango and papaya anthracnose after the fruits are harvested (Li *et al*. 2003).

S. marcescens is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria and have been shown to produce multiple chitinases from different genes (Suzuki *et al*. 2002), and the efficient degradation of chitin is assumed to be achieved by the combined action of the multiple chitinases. Transgenic tobacco plants expressing high levels of *S. marcescens ChiA* exhibited increased tolerance to *R. solani* compared to untransformed control plants (Howie *et al*. 1994). Di Maro *et al*. (2010) exploited tobacco for the production of a biologically active recombinant *Autographa californica* nucleopolyhedrovirus chitinase A (r*ChiA*), as such species is an

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alternative to traditional biological systems for large-scale enzyme production. The comparison between the biochemical and kinetic properties of the r*ChiA* with those of a commercial *S. marcescens* chitinase A indicated that r*ChiA* was thermostable and more resistant at basic pH, two positive features for agricultural and industrial applications.

Shakhbazau *et al*. (2008) studied the pleiotropic effects of the chitinase gene from *Serratia plymuthica* in transgenic potato. Transgenic plants constitutively expressing class I chitinases, in many cases, exhibited higher levels of resistance to fungal infection or delayed development of disease symptoms when compared with wild-type plants (Lee *et al*. 2007). Hence, in the present investigation, we targeted to characterize the *ChiA* from native isolate of *S. marcescens* 141 by expressing in *Escherichia coli* and tobacco plant system.

MATERIALS AND METHODS

The present study was carried out to clone *ChiA* gene from a chitinase efficient isolate of *S. marcescens* to study its expression in *E. coli* and in model plant. The materials used and methods employed are as follows.

Isolates of *S. marcescens* (5, 29, 63, 74, 91, and 141) were obtained from the culture collection of the Department of Agricultural Microbiology, UAS Dharwad. Some of the isolates were from soils of Western Ghats of Uttara Kannada district of Karnataka and were screened for their chitinolytic activity using media containing colloidal chitin as a sole carbon source.

Screening of different isolates for chitinolytic activity

The chitinolytic activity of the available *S. marcescens* isolates were scored by their ability to produce a halo of clearing zone on colloidal chitin plate, colloidal chitin plate was prepared by the modified method of Roberts and Selitrennikoff (Takiguchi 1991). Individual isolates were inoculated on these plates and incubation at 28 ± 2°C for 48 hrs. Those strains can able to produce higher zone on colloidal chitin plate and that was consider as efficient strain to produce potential chitinase enzyme and that strain is used for isolating and cloning of *ChiA* gene.

Cloning and sequencing

The total DNA was isolated from *S. marcescens* isolates by following the protocol of Sambrook and Russell (2001) with some modification. PCR Cloning of the *ChiA* gene was performed by the T/A cloning method following user's manual (MBI Fermentas) using *ChiA* specific primers. *ChiA* gene specific primers were designed for reported *ChiA* gene sequence (Accession No: AB015996) by using Gene Tool Lite Software and synthesized at Bangalore Genei Pvt. Ltd. (Bangalore, India): Chi-A-F (5' GCC CATGGAAGGAATCAGTTATGCGCAAAT 3') and Chi-A-R (5' GCGGATCCCAACGCACTGCAACCGATTAT 3').

PCR was carried out in a Eppendorf Master Cycler gradient in 25 µl reaction volume containing 100 ng DNA template, 3.0 mM $MgCl_2$, 5 pmole of each primer, 2.5 mM dNTPs, 1X *Taq Pol* buffer and 1U *Taq* DNA polymerase (Bangalore Genei) and with 95°C for 4 min. Initial denaturation followed by 40 cycles of 95°C denaturation for 1 min, 55°C annealing for 1 min and 72°C extension for 1 min with a 72°C final extension for 20 min.

The PCR amplicon of *ChiA* (about 1.7 kb) was ligated with linear pTZ57R/T, and these clones were confirmed by PCR amplification and by restriction analysis.

The full length *ChiA* 1.7 kb amplicon cloned in pTZ257R/T was sequenced using M13 forward and reverse primers employing walking technique at Bangalore Genei. The sequences were subjected to analysis using BLAST algorithm available at http://www.ncbi.nim.nih.gov.

Prokaryotic expression

The *ChiA* gene was subcloned into pET28a+ expression vector following a standard recombinant technique downstream to T7 promoter. This ligated mixture was transformed into competent *E. coli* BL21 (DE3) pLysS for expression study. An overnight grown culture of recombinant clones were diluted to 1:100 in fresh LB broth containing kanamycin (Kan; 50 µg/ml) and growth at 37°C for 2 hrs of 0.5 OD and induced by adding isopropyl β-thio-*O*-galactopyramoside (IPTG, 0.5 mM) was added and growth continued for 5 hrs. The cells were then lysed and expression of recombinant protein was studied by SDS-PAGE.

Subcloning into plant expression vector

The plasmid DNA of pNKK0901 (construct containing the *ChiA* insert) and the plant transformation vector pHS100 was isolated and restricted with *Xba*I and *Bam*HI to facilitate directional cloning. *ChiA* was ligated to pHS100 (pNKK0205) was confirmed by PCR amplification and restriction analysis.

Transformation of tobacco

The confirmed recombinant clones of pHS100 was immobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating technique using pRK2013 helper plasmid. The *Agrobacterium* containing recombinant plasmid (pNKK0205) was used for tobacco transformation by using protocol mentioned by Hooykaas *et al*. (1992) with some modification. *Nicotiana tabacum* cv. 'Kanchun' leaves (5-6 week old plants) were surface sterilized by rinsing them for 1 min in 0.1% mercuric chloride. The leaves were cut into pieces of 1 to 2 cm^2 and cocultivated in acetosyringone (200 µM) treated. *Agrobacterium* culture (0.5-0.6) for 20 min for agroinfection. These explants were blotted dried and cocultivated on solid Murashige and Skoog (MS) medium for 2 days. Leaf explants were transferred to MS medium with β-naphthalene acetic acid (0.5 mg/L), 6-benzyladenine (1 mg/L), cephotaxime (Cef; 200 mg/L), Kan (50 mg/L) and incubated at 25 ± 2°C, RH-60% and 16 hrs photoperiod for callus induction. Subculturing was done at 10 days interval using fresh MS medium containing NAA (0.5 mg/L), BAP (1 mg/L), Cef (200 mg/L) and Kan (100 mg/L). The excised calli were placed on shooting medium containing NAA (0.05 mg/L), BAP (1 mg/L), Cef (200 mg/L) and Kan (100 mg/L). The shoots were transferred to MS medium with 100 mg/L Kan, 200 mg/L Cef and no plant growth regulators for rooting. The putative transformants were selected on 100 mg/L Kan, transferred to peat for hardening and DNA was extracted from putative transgenic tobacco plants by a rapid method and checked for the presence of the insert using gene-specific primers.

Transgene expression analysis

PCR positive plants were subjected to single step reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from transgenic plants and control tobacco plants were isolated using RNeasy Plant Mini Kit (Qiagen Co., GmbH, Germany) and subjected to one step RT-PCR using cMasterTM RTplus PCR System and cMasterTM RT Kit (Eppendorf, Hamburg, Germany). An equal amount of total RNA from both transgenic and control tobacco plants were used for this study. The procedure does not allow sample cross contamination and all cDNA is utilized as a template for the PCR step. For one step RT-PCR, specific primers for *Chi*A were used for amplification of the *ChiA* transcript. The amplicon intensity was taken as a measure of transgene expression at transcription level.

Southern blot analysis

Southern blot analysis was performed as described by Southern (1979) with some modifications, to check the copy number of insertion. About 5 g of total DNA was digested with *Eco*RI, size fractioned on 0.8% agarose gel, and transferred to positively charged nylon membrane (Nytron) following the manufacturer's instructions (New England Biolabs, UK). The blots were hybridized to DIG-labelled *npt*II DNA probe and washed to stringency of 0.5x SSC, 0.1% SDS at 68°C. Pre-hybridization and hybridization at high stringency and immunological detection were completed using the DIG-DNA labelling and detection kit (Roche Diagnostics, Germany) according to the manufacturer's instruc-

tions. Following colorometric detection, the gel was photographed and documented.

RESULTS

Screening of isolates for chitinolytic activity When the different isolates of *S. marcescens* were inoculated on colloidal chitin media and incubated at 37°C for 52 hrs the isolate 141 was cleared zone of about 12.5 mm diameter (**Fig. 1**), where as others were found to be cleared zone of less than 12.5 mm diameter this indicates that the isolate 141 has higher efficiency to produce Chitinase.

Cloning and sequencing of the *ChiA* gene

The PCR was carried out using specific primers designed using reported *S. marcescens* nucleotide sequence from the database. pTZ57R/T was used as cloning vector for cloning amplified fragment of a 1691-bp *ChiA* amplicon (**Fig. 2**). The recombinant molecules i.e., pNKK0901 was transferred into *E. coli* DH5α using 5 µl of ligation mixture each and confirmed by PCR and restriction analysis (**Fig. 3**). Sequencing was done by sending to Bangalore Genei. *ChiA* gene sequencing showed 99% homology with reported sequences both at nucleotide and protein level. This sequence as deposited in the NCBI gene bank on 4th Sept 2006 and was accepted on the23rd Sept 2006 (Accession No: DQ990373).

Prokaryotic expression of the *ChiA* gene

The *ChiA* gene was sub-cloned into prokaryotic expression vector pET28a+, confirmed by PCR and restriction digestion (**Fig. 4**). This recombinant plasmid was named as pNKK2603 and transformed into *E. coli* BL21 (DE3) pLySS. Analysis of total protein from transformants showed expression was analyzed on 12% SDS-poly-acrylamide gels. A unique ~58.0 kDa protein in IPTG-induced cultures was observed as compared to control (**Fig. 5**).

Expression studies by chitin plate assay

SDS-PAGE confirmed clones carrying *ChiA*, which were grown on media containing 0.5% colloidal chitin as a sole carbon source. A clear zone was observed around the *ChiA* clone (pNKK2603), but there was no such zone around the control (*E. coli* with pET-28), when incubated at 37°C for three days (**Fig. 6**).

Development of plant expression vector

The *ChiA* gene was released from pNKK0901 and directionally cloned into pHS100 using *Xba*I and *Bam*H1 sites. Plasmid DNA isolated from these transformants showed positive for PCR and restriction analysis and named as pNKK0205 (**Fig. 7**).

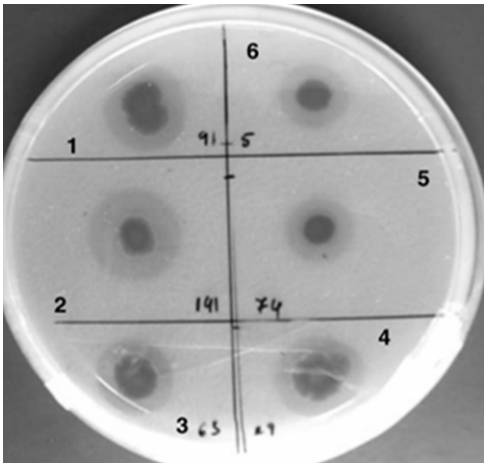

Fig. 1 Chitinolytic activity of native isolates of *Serratia marcescens* on colloidal chitin media. 1. *S. marcescens* 91. 2. *S. marcescens* 141. 3 *S. marcescens* 63. 4. *S. marcescens* 29. 5. *S. marcescens* 74. 6. *S. marcescens* 6.

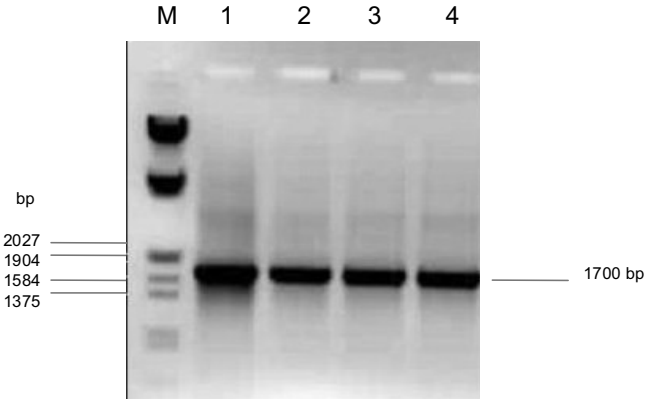

Fig. 2 PCR amplification of *chiA* gene from genomic DNA. M = marker (λDNA/*Eco*RI + *Hind*III). Lanes 1-4 = *S. marcescens ChiA*-specific primer at 55°C.

Transfer of recombinant plant expression vector to *A. tumefaciens* LBA4404

The construct pNKK0205 was mobilized into *A. tumefaciens* LBA4404 through tri-parental mating using *E. coli* containing pRK2013 as helper plasmid. The transconjugants were picked on YEMA containing Kan (50 µg/ml), streptomycin (100 µg/ml) and rifampicin (25 µg/ml) from Himedia Pvt. Ltd. India. The recombinants (pNKK0205) were confirmed through PCR restriction analysis.

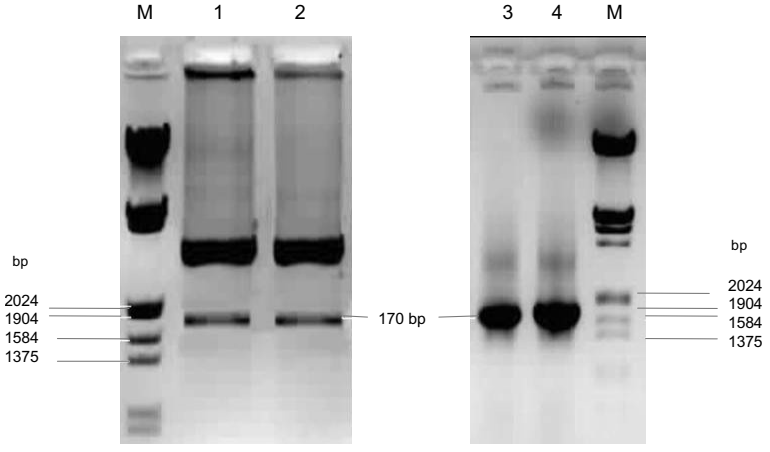

Fig. 3 Restriction and PCR analysis of pNKK0901. M = marker (λDNA/*Eco*RI + *Hind*III). Lanes: 1. Restriction digestion of pNKK0901; 2. Restriction digestion of pNKK0901; 3 and 4. PCR with *ChiA*-specific primer.

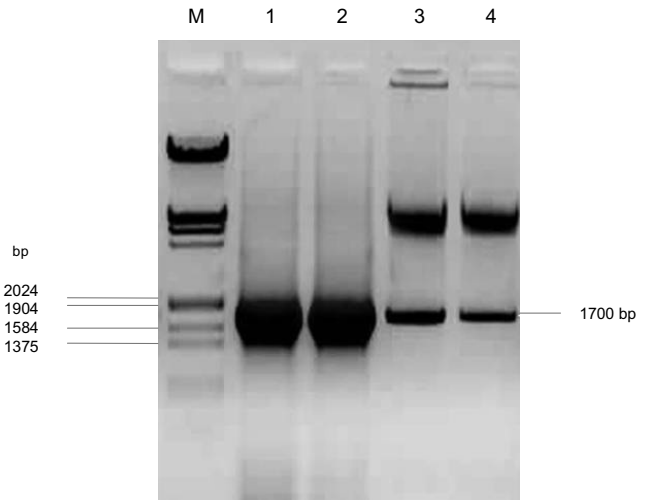

Fig. 4 Restriction and PCR analysis of pNKK 2603. M = marker (λDNA/*Eco*RI + *Hind*III). Lanes: 1 and 2. PCR of pNKK2603 with *ChiA*-specific primer; 3 and 4. Restriction digestion of pNKK 2603 using *Nco*I and *Eco*RI.

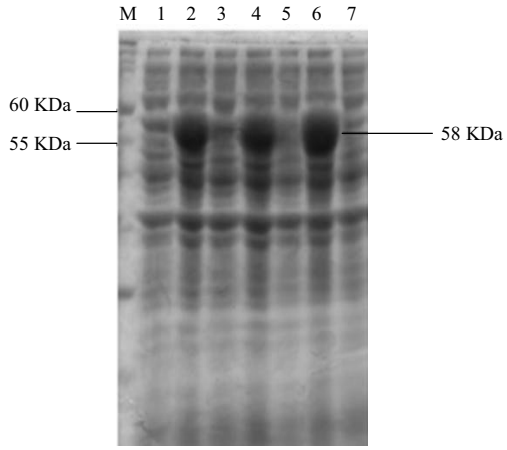

Fig. 5 SDS PAGE analysis of pNKK2603. M. Broad range protein marker. Lanes: 1. Induced BL21(pET28a(+)). 2. Induced pNKK02603A. 3. Uninduced pNKK02603A. 4. Induced pNKK02603B. 5. BL21. 6. Induced pNKK02603C. 7. Uninduced BL21(pET28a(+)).

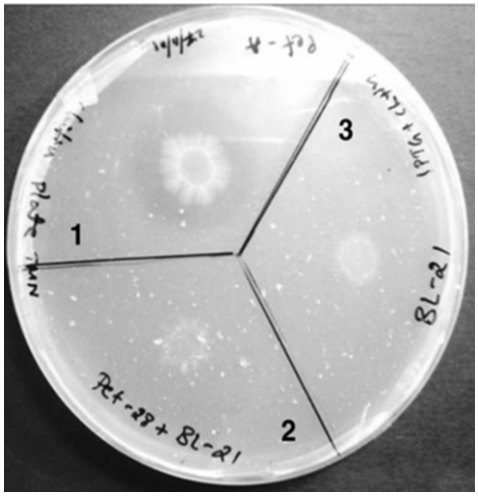

Fig. 6 Chitinase enzyme analysis of pNKK2603. 1. Induced pNKK02603. 2. Induced BL21(pET28a(+)). 3. Induced BL21.

Development of transgenic tobacco plants

Tobacco explants were cocultivated with *A. tumefaciens* transformed pNKK0205 and incubated for 2 days in the dark. The treated explants showed callus and direct shoot induction within 3 weeks (**Fig. 8A**). The majority of shoots and calli transferred to shooting medium turned white on both callus induction and shooting medium. Surviving green shoots having well developed root system (**Fig. 8B**) were transferred to sterilized peat and shifted to green house and hardened (**Fig. 8C**). DNA was isolated from putative transformants and checked for the presence of insert (**Fig. 9**). Two of the four plants were PCR positive for the *ChiA* gene.

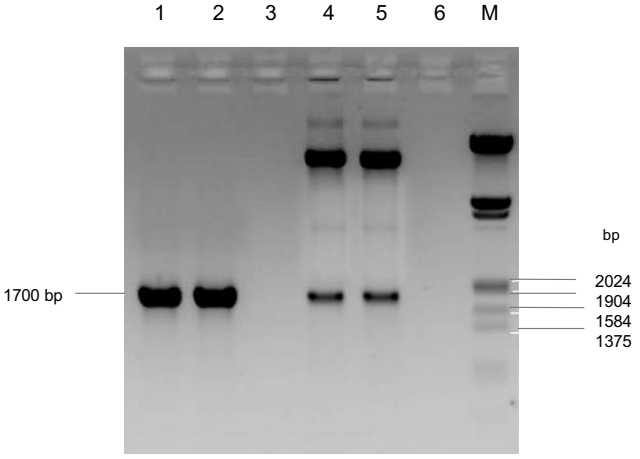

Fig. 7 PCR and restriction analysis of pNKK0205. M. λDNA/*Eco*RI + *Hind*III marker. PCR Lanes: 1. pNKK0205A. 2. pNKK0205B. 3. pHS100. Restriction lanes: 4. pNKK0205A. 5. pNKK0205B. 6. Blank.

Fig. 8 Development of transgenic plants. (**A**) Callus and direct shoot regeneration. (**B**) Plants in rooting media. (**C**) Putative transgenic tobacco plants with *ChiA* gene in tobacco.

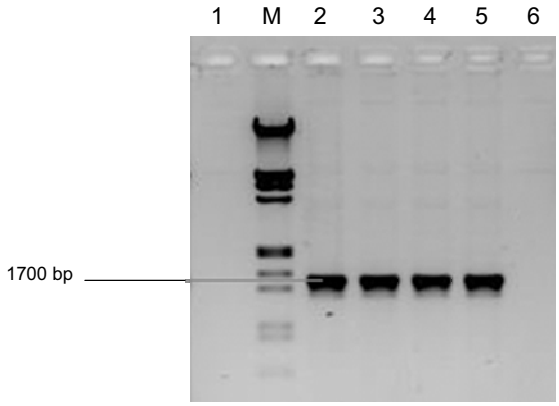

Fig. 9 PCR conformation of putative transgenic plants. M. λDNA/*Eco*RI + *Hind*III marker. Lanes: 1. Negative control (untansformed tobacco plants). 2. *S. marcescence* genomic DNA. 3. Putative tobacco plant 1. 4. Putative tobacco plant 2. 5. Putative tobacco plant 3. 6. Putative tobacco plant 4.

Transgene expression analysis

Equal amount of RNA from transgenic and control was subjected to one-step RT-PCR analysis using *ChiA*-specific primers. RT-PCR amplicon showed 1700 bp in transgenics and was absent in control plants (**Fig. 10**) indicating expression of the transgene. To assess the number of unique transformation events among the PCR-positive plants, DNA gel

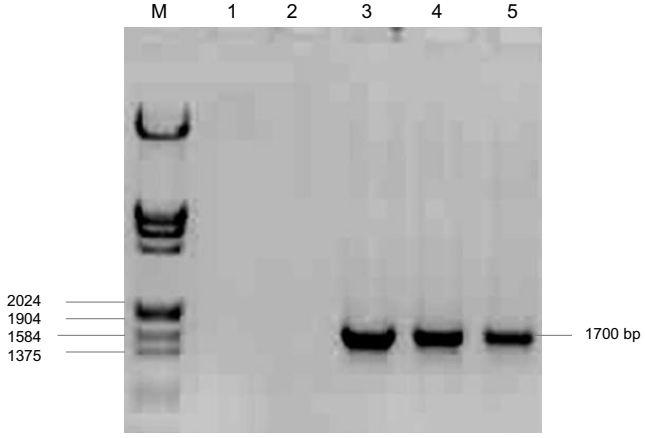

Fig. 10 RT-PCR conformation of putative transgenic plants. M. λDNA/*Eco*RI + *Hind*III marker. Lanes: 1. Negative control (untransformed tobacco plants). 2. Transgenic plant 4. 3. Transgenic plant 1. 4. Transgenic plant 2. 5. Transgenic plant 3.

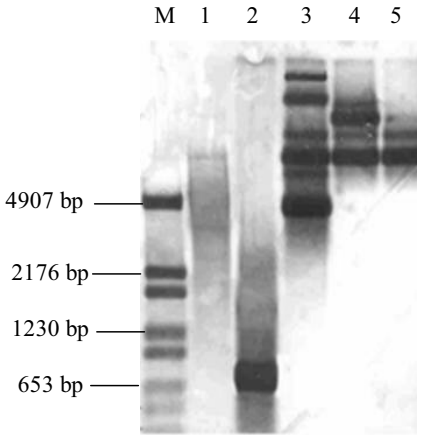

Fig. 11 Southern blot confirmation of putative transgenic plants. M. λDNA/*Eco*RI + *Hind*III marker. Lanes: 1. Negative control (untransformed tobacco plants). 2. Positive control (pNKK0205 plasmid DNA). 3. Transgenic plant 1. 4. Transgenic plant 2. 5. Transgenic plant 3.

blot analysis was performed. Southern blot analysis of T_0-transgenic plants showed hybridization signals with the *npt*II probe in both the PCR positive plant tested but was absent in control plants (**Fig. 11**).

DISCUSSION

The success of *S. marcescens* as a biocontrol agent is believed to involve many factors and diverse modes of action. The hydrolytic enzymes (e.g. protease and chitinase) produced *S. marcescens* likely play a key role in its ability to penetrate and kill a host. Chitinases are class of hydrolytic enzymes that catalyze the hydrolysis of the β-1,4-*N*-acetyl-D-glucosamine linkages in chitin and chitodextrins (Kasprzewska 2003), and play an important role in decomposing chitin and as an antifungal agent. *S. marcescens* is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. In this study, we cloned a chitinase gene from *S. marcescens* 141 as it showed higher level of chitinase A activity among six strains of *S. marcescens* that were screened for their chitinolytic activity by growing on colloidal chitin plate in which chitin was the sole carbon source. Similar work was done by Zarei *et al.* (2010) in which chitinase production by newly isolated *S. marcescens* B4A was optimized following Taguchi's array methods. Twenty-three bacterial isolates were screened from shrimp culture ponds in the South of Iran. A chitinase-producing bacterium was isolated based on its ability to utilize chitin as the sole carbon source. The 1.7-Kb amplicon was cloned into linearised pT257R/T and transformants were screened through a blue-white screening assay. The *Chi*A sequences of 141 are very closely related to those of a previously reported *Chi*A gene of *S. marcescens* strains in terms of amino acid and nucleotide sequences. Okay (2008) isolated the *ChiA* gene from a highly chitinolytic local isolate of *S. marcescens* and expressed in an anti-Coleopteran *Bacillus thuringiensis* and comparison of the characteristics of native and recombinant *ChiAs*. pTZ57R is more of a cloning vector, these *ChiA* gene was further subcloned into a prokaryotic expression vector pET28a(+), to study their expression in *E. coli* BL21 (plysS). The recombinant clones were analysed by PCR amplification and restriction and named as pNKK2603.

SDS-PAGE was carried out for recombinant *E. coli* containing pNKK2603 by induction with IPTG (Watanabe *et al*. 1997), which showed expression of 58.5 KDa protein. The size of expressed chitinase *A* protein of *S. marcescens* 141 observed upon induction is similar to that reported by Wu *et al*. (2009) who cloned the *S. marcescens chiA* gene by PCR. The complete gene was constructed into a pRSET vector and expressed in *E. coli* the molecular mass of the protein was 58,607 Da, consistent with a theoretical calculation of the deduced protein without the signal peptide. The recombinant enzyme was characterized and tested for the preparation of chitobiose. Further expression studies *chiA* gene was done by chitin plate assay, there was a clearing zone was formed around the *chiA* clone (pNKK2603). But there was no such zone around the control. Further, the *chiA* gene was directionally cloned into a plant transformation vector pHS100, under CaMV35S tandem promoter at *Xba*I and *Bam*HI sites. The ligated product was transferred into *E. coli* DH5α. The recombinant pHS100 clones obtained were confirmed for the *chiA* insert by size through PCR amplification and restriction digestion with *Xba*I and *Bam*HI and named as pNKK0205. The confirmed clones containing construct pNKK0205 were transferred to *A. tumefaciens* LBA4404 by triparental mating. The *Agrobacterium* with the pNKK0205 construct was used to transform tobacco by following the protocol from Hooykaas and Suchilperoort (1992). The plants, which rooted on medium with 200 µg/ml Kan, were checked for the presence of inserts and more than 50% of plants were PCR-positive. Transgene expression was checked by one-step RT-PCR. An RT-PCR amplicon of 1700 bp was observed in transgenics and no band was seen in control plants, which implied the expression of the transgene at the transcription level. Dana *et al*. (2006) generated transgenic tobacco (*Nicotiana tabacum*) lines that overexpressed the endochitinases CHIT33 and CHIT42 from the mycoparasitic fungus *Trichoderma harzianum* and confirmed their result by PCR, RT-PCR and Western blot analysis. The intensity of the amplicon is a direct indication of transcription of the transgene. Further, to find the presence and number of copies in the transformed plants, Southern blot hybridization was carried out using an *npt*II-specific probe. The transgenic tobacco plant 1 shown to be having the 3 copies of *ChiA* gene followed by two copies in transgenic plant 2 and one copy of the *chiA* gene in transgenic plant 3 (**Fig. 11**). Ganesan *et al*. (2009) described a simple and reproducible protocol for transgenic cotton regeneration and characterization of chitinase (*Chi*II) gene expression against two different fungal pathogens in cotton and was identified one to six copies of chitinase transgene in rice plant through Southern blot analysis. However, there is a need to do a detailed analysis of gene expression in these plants by a bioassay to challenge infection by pathogenic isolates of tobacco fungal diseases like *Fusarium oxysporum* and *Phytophthora nicotianae*.

The utility of *S. marcescens* (Howie *et al*. 1994) and other chitinases has been demonstrated using transgenic tobacco. Lund *et al*. (1989) investigated the modifications and the cellular location of *ChiA* when expressed in transgenic tobacco plants. Immunoblots on total leaf protein probed with antibody to *ChiA* showed that when the bacterial chitinase is expressed in plants was migrated in a series of discrete bands with either the same or a slower mobility than

the secreted bacterial protein. These results show that a bacterial signal sequence can function in plant cells, and that protein secretion from plant cells probably operates by a default pathway. Transgenic *indica* rice expressing a bitter melon (*Momordica charantia*) class I chitinase gene (McCHIT1) conferred enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani* (Li *et al.* 2009) while transgenic Indian cotton (*Gossypium hirsutum*) harboring the rice chitinase gene (*Chi*II) conferred resistance to two fungal pathogens *Fusarium oxysporum* and *Alternaria macrospora*. *S. marcescens ChiA* gene increased tolerance to *R. solani* (Howie *et al*. 1994), and the *Saccharomyces cerevisiae* CTCI gene showed decreased susceptibility ranging from 50-70% to *Botrytis cinerea* in transgenic tobacco. The usefulness of the cloned *ChiA* construct developed during our study needs to be explored in specific crops and checked against *R. solani* and other phytopathogenic fungi. Microbes are a good source of genes for chitinases. It is necessary to clone a large number of such genes from different sources and also effective studies could be done on different pathogens through Bioassy which can be deployed in crop plants.

REFERENCES

Bird AF, McClure MA (1976) The tylenchid (nematode) egg shell: Structure, composition and permeability. *Parasitology* **72**, 19-28

Chernin L, Ismailov Z, Haran S, Chet I (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Applied and Environmental Microbiology* **61**, 1720-1726

Dana MM, Pintor TJA, Cubero B (2006) Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. *Plant Physiology* **142**, 722-730

Di Maro A, Terracciano I, Sticco L, Fiandra L, Ruocco M, Corrado G, Parente A, Rao R (2010) Purification and characterization of a viral chitinase active against plant pathogens and herbivores from transgenic tobacco. *Journal of Biotechnology* **147**, 1873-4863

Frankowski J, Lorito M, Scala F, Schmid R, Berg G, Bahl H (2001) Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Archives of Microbiology* **176**, 421-426

Ganesan M, Bhanumathi P, Ganesh Kumari K, Lakshmi Prabha A, Song P, Jayabalan N (2009) Transgenic Indian cotton (*Gossypium hirsutum*) harboring rice Chitinase gene (*Chi*II) confers resistance to two fungal pathogens. *American Journal of Biochemistry and Biotechnology* **5**, 63-74

Gomes RC, Semedo LT, Soares RM, Linhares LF, Ulhoa CJ, Alviano CS, Coelho RR (2001) Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *Journal of Applied Microbiology* **90**, 653-661

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280**, 309-316

Hooykaas PJ, Schilperoort RA (1992) *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology* **19**, 15-38

Horn SJ, Sobotten A, Synstad B, Sikorski P, Solie M, Varum KM, Eijsink VGH (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. *FEBS Journal* **273**, 491-503

Howie W, Joe L, Newbigin E, Suslow T, Dunmuir P (1994) Transgenic tobacco plants which express the *ChiA* gene from *Serratia marcescens* have enhanced tolerance to *Rhizoctonia solani*. *Transgenic Research* **3**, 90-98

Kasprzewska A (2003) Plant chitinases regulation and function. *Cellular and Molecular Biology Letters* **8**, 809-824

Kirubakaran SI, Sakthivel N (2007) Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli*. *Protein Expression and Purification* **52**, 159-166

Kitamura E, Kamei Y (2003) Molecular cloning sequencing and expression of the gene encoding a novel chitinase A from a marine bacterium *Pseudomonas* sp. PE2 and its domain structure. *Applied Microbiology and Biotechnology* **61**, 140-149

Lee YS, Park IH, Yoo JS, Chung SY, Lee YC, Cho YS, Ahn SC, Kim CM, Choi YL (2007) Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. *Bioresource Technology* **98**, 2734-2741

Li P, Pei Y, Sang X-C, Ling Y-H, Yang Z-L, He G-H (2009) Transgenic *indica* rice expressing a bitter melon (*Momordica charantia*) class I chitinase gene (McCHIT1) confers enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani*. *European Journal of Plant Pathology* **125**, 533-543

Li YC, Chang CT, Hsiao ES, Hsu JS, Huang JW, Tzen JT (2003) Purification and characterization of an antifungal chitinase in jelly fig (*Ficus awkeotsang*) achenes. *Plant Cell Physiology* **44**, 1162-1167

Lund P, Lee RY, Dunsmuir P (1989) Bacterial Chitinase is modified and secreted in transgenic tobacco. *Plant Physiology* **91**, 130-135

Masuda S, Kamada H, Satoh S (2001) Chitinase in cucumber xylem sap. *Bioscience, Biotechnology and Biochemistry* **65**, 1883-1885

Monreal J, Reese E (1969) The chitinase of *Serratia marcescens*. *Canadian Journal of Microbiology* **15**, 689-696

Okay S, Tefon BE, Ozkan M, Ozcengiz G (2008) Expression of chitinase A (*ChiA*) gene from a local isolate of *Serratia marcescens* in Coleoptera-specific *Bacillus thuringiensis*. *Journal of Applied Microbiology* **104**, 161-170

Reyes-Ramírez A, Escudero-Abarca BI, Aguilar-Uscanga G, Hayward-Jones PM, Barboza-Corona JE (2004) Antifungal activity of *Bacillus thuringiensis* chitinase and its potential for the biocontrol of phytopathogenic fungi in soybean seeds. *Journal of Food Science* **69**, 131-134

Shakhbazau AV, Yakovleva GA, Rod'kina IA, Kartel NA (2008) Pleiotropic effects of the Chitinase gene from *Serratia plymuthica* in transgenic potato. *Cytology and Genetics* **42**, 75-80

Suzuki K, Sugawara N, Suzuki M, Uchiyama T, Watanabe T (2002) Chitinase A, B and C1 of *Serratia marcescens* 2170 produced by recombinasnt *Escherichia coli*; Enzymatic properties and synergism in chitin degradation. *Bioscience, Bacteriology and Biochemistry* **66**, 1075-1083

Takiguchi Y (1991) Physical properties of chitinous materials. *Advances in Chitin Science* **111**, 1-7

Trifonova EA, Komarova ML, Syrnik OA, Kochetov AV, Shumnyi VK (2002) Transgenic tobacco (*Nicotiana tabacum* SR1) plants expressing the gene coding for *Serratia marcescens* nuclease. *Genetika* **38**, 274-277

Vaaje G, Horn JS, Daan MF, van A, Bjørnar S, Vincent G, Eijsink H (2005) The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *The Journal of Biological Chemistry* **280**, 28492-28497

Veldkamp H (1955) A study of the aerobic decomposition of chitin by microorganisms. *Mededelingen Landbouwhogesch Wageningen* **55**, 127-174

Wang FP, Li Q, Zhou Y, Li MG, Xiao X (2003) The C-terminal module of Chi1 from *Aeromonas caviae* CB101 has a function in substrate binding and hydrolysis. *Proteins* **53**, 908-916

Wang SL, Wang H (2000) Microbial reclamation of shellfish wastes for the production of chitinases enzyme. *Microbial Technology* **28**, 376-382

Watanabe T, Kimura K, Sumiya T, Nikaidou N, Suzuki K, Taiyoji M, Ferrer S, Regue M (1997) Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *Journal of Bacteriology* **179**, 7111-7117

Wu Y-J, Cheng Y-C, Li Y-K (2009) Cloning and expression of Chitinase A from *Serratia marcescens* for large-scale preparation of *N,N*-diacetyl chitobiose. *Journal of the Chinese Chemical Society* **56**, 688-695