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Agrobacterium-mediated transformation of chitinase gene from the actinorhizal tree *Casuarina equisetifolia* in *Nicotiana tabacum*

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Abstract: Genetic transformation of plants offers the possibility of testing hypotheses on the function of individual genes and enables exploration of transgenes for targeted trait improvement. Cloning of the full-length class I chitinase from the *Casuarina equisetifolia* (*CeChi1*) was earlier reported by our team. In the present study, tobacco was used as a model system to functionally evaluate the potential of *CeChi1* driven by ubiquitin promoter. The *pUH-CeChi1* construct was introduced into tobacco by *Agrobacterium*-mediated transformation and the putative transformants were confirmed for stable gene integration, transgene expression and recombinant protein production using PCR, RT-qPCR, antifungal assays and *in planta* analysis. The *in vitro* antifungal bioassay using the total proteins from leaves of transformed plantlets revealed the characteristic lysis of hyphal tips of pathogenic fungi including *Trichosporium vesiculosum*, *Fusarium oxysporum* and *Rhizoctonia solani*. The *in planta* bioassay of transformed tobacco showed reduced disease symptoms when compared to untransformed wild plants. The study revealed that the class I chitinase isolated from *C. equisetifolia* can act as a potential gene resource in future transformation programs for incorporating disease tolerance.

Key words: chitinase; functional validation; pathogenesis-related protein; transformation; tobacco.

Abbreviations: BAP, benzylaminopurine; *hph*, hygromycin phosphotransferase; IBA, indole butyric acid; MS, Murashige and Skoog; NAA, naphthalene acetic acid; PMSF, phenylmethyl sulfonyl fluoride; PR, pathogenesis-related; RT-qPCR, quantitative real-time PCR.

Introduction

Chitin, one of the most abundant biopolymer in nature next to cellulose and lignin, is a major structural component of the fungal cell wall. Plant chitinases are hydrolytic enzymes (EC 3.2.1.14) that hydrolyze chitin, chitosan, lipochitooligosaccharides, peptidoglycan, arabinogalactan and glycoproteins containing N-acetylglucosamine (Grover 2012). Genome-wide analysis revealed that chitinases are represented in large multi-gene families and expressed under diverse conditions. In *Oryza sativa* and *Populus trichocarpa*, 37 members of chitinases have been reported, while in *Arabidopsis*, 24 genes were documented in the genome (Xu et al. 2007; Jiang et al. 2013). They are grouped under pathogenesis-related (PR) protein families PR3, PR4, PR8 and PR11, and play an important role in plant defence against infection by pathogens (Neuhaus 1999). The induction of chitinases during systemic acquired resistance is well characterized and purified chitinases show antifungal activity under *in vitro* conditions (Schlumbaum et al. 1986; Leah et al. 1991). The role of chitinases in plant defence has been extensively reviewed (Sharma et al. 2011; Grover 2012; Veluthakkal et al. 2012; Hamid et al. 2013). Ectopic expression of chitinases in transgenic systems has shown enhanced disease resistance in several species (Yamamoto et al. 2000; Dana et al. 2006; Maximova et al. 2006; Xiao et al. 2007; Kovács et al. 2013).

Tobacco is the most commonly used model plant system in transgenic research and is an ideal organism for the study of basic biological functions, like plant-pathogen interactions, environmental responses, growth regulation and senescence (Ger et al. 2002; Jube & Borthakur 2007; Swathi et al. 2008). In the present study, the class I chitinase (*CeChi1*) isolated from the needle tissues of *Casuarina equisetifolia* challenged with fungal elicitors (Veluthakkal & Dasgupta 2012) was ectopically expressed in tobacco, to functionally validate the antifungal property of the encoding protein.

Material and methods

Plant materials and culture condition

Seeds of *Nicotiana tabacum* cv. *samsun* were surface-sterilized with 30% (v/v) sodium hypochloride for 20 min,

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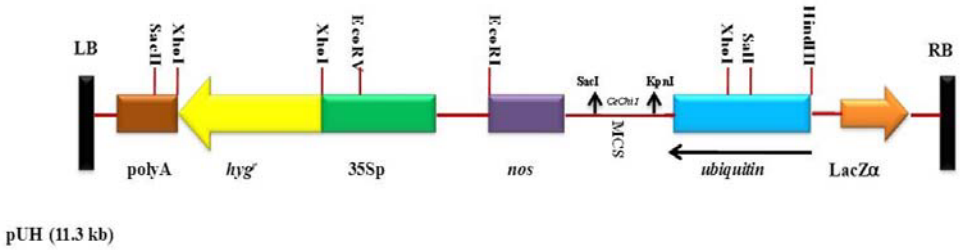

Fig. 1. Schematic representation of *pUH-CeChi1* construct.

rinsed 3-times with sterile distilled water and germinated on half strength Murashige and Skoog (MS) media (Himedia, India) in a growth chamber at 25±2℃ with 16 h light and 8 h dark photo-cycle for a period of one month. The meristematic tissues of the emerging shoots were cut and maintained in MS medium containing 1.0 mg/L benzylaminopurine (BAP) by sub-culturing every two weeks prior to transformation.

Agrobacterium strain, plasmid and bacterial culture
Agrobacterium tumefaciens strain LBA4404 bearing the binary vector *pUH-CeChi1* (Katiyar-Agarwal et al. 2002) was used for transformation studies. The T-DNA region of the binary vector contained a hygromycin phosphotransferase gene (*hph*) under the control of the CaMV 35S promoter and *CeChi1* gene under the control of the maize ubiquitin-1 promoter (Fig. 1). *Agrobacterium* strain LBA4404 harbouring *pUH-CeChi1* were grown in YEP broth with 10 mg/L rifampicin and 50 mg/L kanamycin and incubated at 28℃ with constant shaking at 250 rpm for 36–48 hours. When the optical density reached 0.6–0.8, cells were pelleted down at 5,000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended in 15 mL of half-strength basal MS medium containing 100 mM acetosyringone.

Leaf disc transformation
A modified transformation method of Öktem et al. (1994), which was first described by Horsch et al. (1985), was used for transforming tobacco leaves. Young leaves from 20–30-day old tobacco plants were used for leaf disc transformation. Leaf discs of 1×1 cm were pre-incubated on pre-incubation media [MS basal medium with 0.1 mg/L naphthalene acetic acid (NAA) and 1 mg/L BAP] containing 100 mM acetosyringone. Approximately, 15–20 discs were incubated at 25±1℃ for 48 h with photoperiod of 16/8 h. Uninfected discs cultured on selection media served as negative control, while leaf discs placed on regeneration media without co-cultivation was used as positive control.

Co-cultivation and regeneration of transformants
Tobacco leaf discs were incubated in the *Agrobacterium* harbouring *pUH-CeChi1* for 25–30 min with gentle shaking at regular intervals under sterile conditions. Subsequently, leaf discs were dried on sterile tissue paper and transferred to pre-incubation medium and incubated in dark for 48–72 h. Further, they were removed from co-culture media and placed on MS selection media containing 300 mg/L cefotaxime and 25 mg/L hygromycin after washing with cefotaxime (250–300 mg/L) to remove excessive bacterial cells. Plates were resealed and incubated for two weeks under the same conditions. Regeneration started after 15-20 days and regenerated leaf discs were transferred to fresh selection medium after every two weeks. When shoots reached 1–2 node stage, they were transferred to the rooting medium [MS media with 0.1 mg/L indole butyric acid (IBA)] with reduced antibiotic concentration. After root formation, the plantlets were transferred to transgenic green house.

Molecular analysis of transgenic plants
Genomic DNA isolation and PCR amplification
Two putative transformants (T1 and T2) were randomly selected from two independent events for further studies. Genomic DNA from leaves of control and putative transgenic tobacco plants were isolated by cetyl dimethyl ethyl ammonium bromide method (Richards 1997). Approximately 100 mg leaf material was taken for isolation and the concentrations were determined spectrophotometrically. Subsequent to genomic DNA isolation, PCR was carried out with specific primer pairs to amplify the transgenes. *CeChi1* was amplified using the primer pair KpnF: 5'-GGAATTCCGGTACCAAAATGAGGTTTTGGATCTT TGC-3' and SacR: 5'-CCCGAGCTCCTACATGGTAT CCACCAAGAGTCCATTG-3', while the selection marker hygromycin was amplified using HygF: 5'-GCTTTCAGC TTCGATGTAGGAG-3' and HygR: 5'-CACGCCATGTAG TGTATTGACC-3'. Primer pairs amplifying the transgene combinations, such as QChiFP: 5'-TGACCCTGTCGTTT CCTTTAAGTC and HygR: 5'-CACGCCATGTAGTGTAT TGACC-3'), and KpnF: 5'-GGAATTCCGGTACCAAAAT GAGGTTTTGGATCTTTGC-3' and HygR: 5'-CACGCC ATGTAGTGTATTGACC-3' were also amplified. A positive control of pure plasmid *pUH-CeChi1* was also analyzed. The PCR reaction contained 50 ng of genomic DNA as template and amplification was performed with 1 min at 95℃, 30 cycles of 30 s at 94℃ for denaturation, 1 min at 55 ℃ for annealing and 1 min at 72℃ for elongation. A final extension was carried out for one cycle of 10 min at 72℃. The amplified PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel.

Analysis of transgene expression
Total RNA was extracted from leaves of untransformed control and one putative transformant (T2) using the Plant RNA Mini kit (Chromous Biotech, Bangalore) according to the manufacturer's instruction. Subsequently, total RNA was treated with RNase free DNase I (Fermentas, USA) according to the manufacturer's protocol and first strand cDNA was synthesized using cDNA synthesis kit (Fermentas, USA). Quantitative real-time PCR (RT-qPCR) was performed to quantify the expression of the *CeChi1* transgene in wild and putative transgenic. The primer pairs (QChiFP: 5'-TGACCCTGTCGTTTCCTTTAAGTC-3' and QChiRP: 5'-TGATGTTCGTAACAACACCGTACC-3') was used to amplify the target gene, while β-actin from tobacco: TactinFP: 5'-CCTGAGGTCCTTTTCCAACCA-3'; TactinRP: 5'-GGATTCCGGCAGCTTCCATT-3' (Schmidt & Delaney 2010) was used as an endogenous control to normalize the data for differences in input RNA and

efficiency of reverse transcription between the different samples. RT-qPCR reaction was performed in fast optical reaction tube (Microamp, Applied Biosystems) using StepOne plus Sequence Detection System (Applied Biosystems, USA) and associated software using the SYBR green chemistry. PCR was performed in a final volume of 10 µL containing 5 µL of 2X SYBR Green Jumpstart Taq Ready Mix for Quantitative PCR (Sigma-Aldrich, USA), 500 nM each of forward and reverse primers and 100 ng of cDNA template. After an initial activation step of DNA polymerase at 94℃ for 2 min, samples were subjected to 40 cycles of amplification (denaturation at 94℃ for 15 s, annealing and extension at 60℃ for 1 min). The melt curves were analyzed at 60–95℃ after 40 cycles. The experiment was performed in triplicate. Non-RT control, where 10 ng of total RNA without reverse transcription was used to monitor genomic DNA contamination and non-template control (NTC; water template), was also used. Changes in gene expression as relative fold difference between transgenic sample and control plantlet was calculated using the comparative Ct (2-ΔΔCt) method (Schmittgen et al. 2000; Livak & Schmittgen 2001).

Extraction of intercellular and intracellular fluids

Intercellular fluid was extracted from leaves of untransformed and putative transformant (T2) according to Rathmell & Sequoia (1974) with few modifications. Freshly collected leaves were cut into pieces of 4–5 cm and infiltrated in vacuum with gentle agitation for three periods of 30 s each, with an excess of cold buffer (4℃) containing 25 mM Tris-HCI, pH 7.8, containing 0.5 M sucrose, 10 mM $MgC1_2$, 10 mM $CaCl_2$, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) and 5 mM 2-mercaptoethanol. Pieces were gently blotted dry, rolled up and placed in a syringe inside a 30 mL centrifugation tube. After centrifugation at $1{,}000\times g$ for 10 min at 4℃, the extract was recovered and used immediately. Leaves were taken from the syringe and ground into fine powder in liquid nitrogen to extract the intracellular fluids. The powder was suspended in the extraction buffer (100 mM sodium acetate, pH 5) in a ratio of 1:3 and mixed by vortexing. The samples were centrifuged at 12,000 rpm for 10 min at 4℃ and the supernatant was used for electrophoresis. The intercellular and intracellular fractions were separated on a 12% SDS PAGE alongside a medium range protein molecular marker with 14.3–97.4 kDa range (Merck Genei, India; catalogue number 623111275001730) and stained overnight with Coomassie Brilliant Blue R-250.

Extraction of total proteins

One hundred mg of fresh leaves were harvested from untransformed and putative transformant (T2) and ground into fine powder in liquid nitrogen. The powder was suspended in the extraction buffer (100 mM sodium acetate, pH 5) in a ratio of 1:3 and mixed by vortexing. The samples were centrifuged at 12,000 rpm for 10 min at 4℃ and the supernatant was used for subsequent bioassay studies.

Determination of antifungal activity of total protein

The fungal isolates of *Fusarium oxysporum*, *Trichosporium vesiculosum* and *Rhizoctonia solani* were maintained in potato dextrose agar medium. Antifungal activity was determined under sterile conditions by hyphal extension inhibition assay. Agar blocks containing fungal mycelia were taken from actively growing plates and inoculated in petri dishes containing potato dextrose agar and incubated at 32℃ for 24–32 h. Subsequently, sterile filter paper discs (1 mm thickness) were placed towards the periphery of the growing hyphae and total proteins isolated from untransformed and T2 were added to the disc at a concentration of 500 µg/mL. A buffer control was also maintained. The plates were incubated for 24 h and observations were made for the appearance of crescent shaped inhibition zone. The growing hyphae from different regions were harvested and observed under microscope to evaluate the effect of the proteins on hyphal morphology. Digital images were used to derive the width and volume of control and treated hyphae using image analysis software Leica QWin 3.0.

In planta biosassay

R. solani was grown on potato dextrose agar medium at 25℃ and after 1 week the mycelial mat was harvested and spore suspension was prepared by flooding the petri dish with sterile water, filtering and adjusting the resulting spore suspension to 2×10^6 spores/mL. Tobacco plants (fifteen each from untransformed and transformant T2) were subcultured onto fresh MS medium without antibiotics and allowed to root. Subsequently, 1 mL fungal suspension was injected into the medium to allow fungal contact with the roots. Mock-inoculation was done by spraying sterile water onto the medium. The plants were then incubated at room temperature for 4 weeks and periodic (daily) observations were made for appearance of disease symptoms.

Results and discussion

Tobacco was used as a model system to functionally characterize *CeChi1* and *Agrobacterium*-mediated transformation procedure was followed due to its well-established protocol and high efficiency in generating transgenic events (Öktem et al. 1994).

Callus induction, selection and regeneration of transformants

Transformed cells grew normally in the presence of hygromycin, while non transformed cells died. Regeneration started as small green calli around the periphery of leaf discs after three weeks on selection media. The hygromycin-resistant calli eventually showed signs of organogenesis and produced small shoots from the calli after 2–3 weeks. Rooting started after 5–6 days of transferring the shoots to rooting media. A number of independent hygromycin-resistant transgenic tobacco lines were generated. All these hygromycin-resistant tobacco plants showed normal phenotype. A total of 250 leaf discs were co-cultured with LBA4404 harbouring *pUH-CeChi1* and 166 leaf discs regenerated on selection media containing hygromycin with 66.4% transformation efficiency (Table 1). Callus formation was observed in positive control leaf discs after 3–4 weeks. Leaf discs in negative control did not yield any calli and necrosis was observed due to hygromycin toxicity.

Molecular analysis of putative transgenic plants

PCR confirmation of transformation events

Integration of the T-DNA into the putative transgenics was confirmed by PCR analysis using primer combinations amplifying *CeChi1* and *hph* transgenes, which specifically amplify in transformed plants. The *CeChi1* and *hph* specific primers amplified at 1.0 kb and 340 bp,

Table 1. Transformation efficiency of LBA4404 harbouring *pUH-CeChi1* in tobacco.

No. of leaf discs infected	No. of discs regenerated on selection media	Transformation efficiency (%)	Total plants produced
250	166	66.4	89

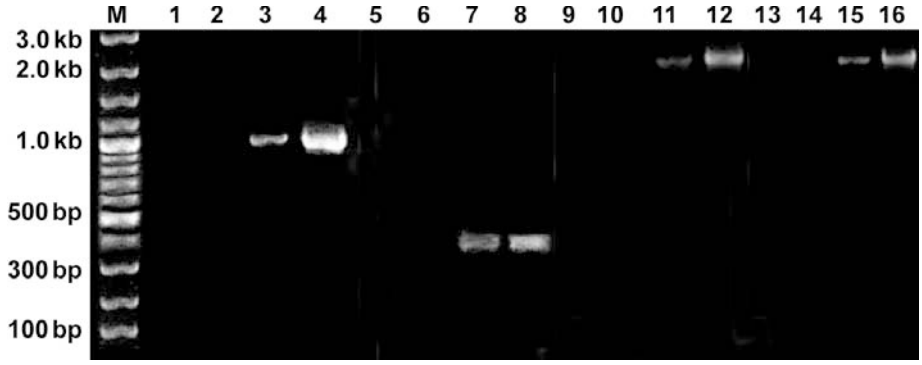

Fig. 2. PCR confirmation of putative transformants using different primer combinations. Lane M: GeneRuler ™ 100 bp plus DNA ladder (Fermentas, USA); lane 1: amplification of genomic DNA from untransformed tobacco with KpnF and SacR primer pairs; lane 2: amplification of genomic DNA from T1 with KpnF and SacR primer pairs; lane 3: amplification of genomic DNA from T2 with KpnF and SacR primer pairs; lane 4: positive control (*pUH-CeChi1* plasmid); lane 5: amplification of genomic DNA from untransformed tobacco with HygF and HygR primer pairs; lane 6: amplification of genomic DNA from T1 with HygF and HygR primer pairs; lane 7: amplification of genomic DNA from T2 with HygF and HygR primer pairs; lane 8: positive control (*pUH-CeChi1* plasmid); lane9: amplification of genomic DNA from untransformed tobacco with QchiFP and HygR primers; lane 10: amplification of genomic DNA from T1 with QchiFP and HygR primers; lane 11: amplification of genomic DNA from T2 with QchiFP and HygR primers; lane 12: positive control (*pUH-CeChi1* plasmid); lane 13: amplification of genomic DNA from untransformed tobacco with KpnF and HygR primers; lane 14: amplification of genomic DNA from T1 with KpnF and HygR primers; lane 15: amplification of genomic DNA from T2 with KpnF and HygR primers; lane 16: positive control (*pUH-CeChi1* plasmid).

respectively, in the putative transformant T2 confirming the transformation event (Fig. 2). Amplification using primer combinations KpnF and HygR, and QChiFP and HygR yielded amplicons of size 2.0 kb and 2.5 kb, respectively, in the putative transformant T2. None of the primer pairs amplified in the putative transgenic line T1 and untransformed wild tobacco DNA (Fig. 2). The results confirmed that the transformation event T2 stably integrated both *CeChi1* transgene and plant selectable hygromycin marker gene. The untransformed condition of T1 could be due to an escape during selection.

Expression analysis of CeChi1 in transformed tobacco
The cDNA synthesized from untransformed tobacco and putative transformant T2 were subjected to RT-qPCR analysis and result indicated a 3.8-fold expression of *CeChi1* in the transformed line T2 in comparison to the untransformed control.

Analysis of intercellular and intracellular proteins
Analysis of the intercellular and intracellular proteins from untransformed and transformant T2 with total protein from untransformed tobacco revealed the overexpression of chitinase protein at ∼31 kDa in intracellular fraction of putative transformant T2 when compared to intracellular fraction of the untransformed tobacco (Fig. 3).

Following genome sequencing of crop plants, one of the major challenges today is determining the function of all the predicted genes/proteins. Genetic transformation of plants offers the possibility of testing hypotheses and providing insight into the function of individual genes and exploration of transgenes for targeted trait improvement (Himmelbach et al. 2007). Several studies on using plant system to functionally characterize genes have been reported (Condori et al. 2009; Yuan et

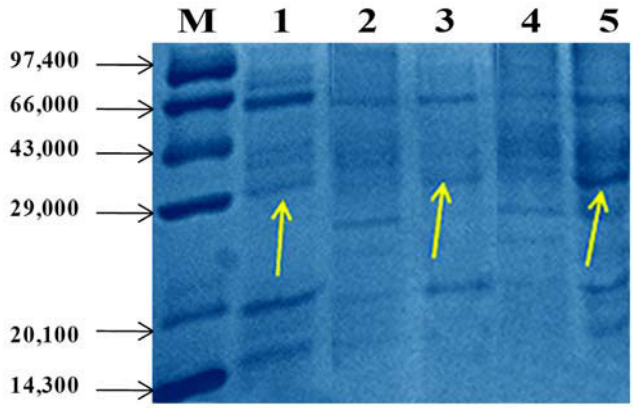

Fig. 3. SDS-PAGE analysis of intercellular and intracellular proteins extracted from untransformed tobacco and putative transgenic line T2. Lane M: molecular weight markers (medium range; Bangalore Genei, India); Lane 1: total protein from untransformed tobacco; lane 2: intercellular protein from untransformed tobacco; lane 3: intracellular protein from untransformed tobacco; lane 4: intercellular protein from transgenic line T2; lane 5: intracellular protein from transgenic line T2. Arrow indicates the presence of CeChi1 at ∼31 kDa. The increased intensity of band (arrow) in the lane 5 indicates the overexpression of *CeChi1* in transgenic tobacco.

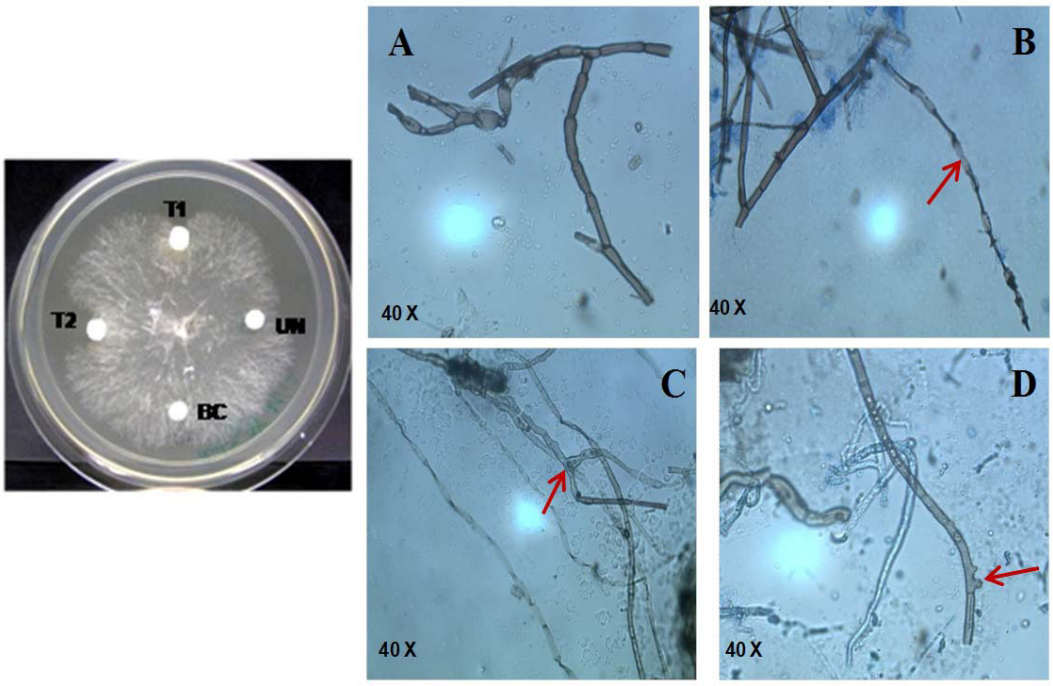

Fig. 4. *In vitro* hyphal morphology studies against *Trichosporium vesiculosum*. Hyphal extension inhibition assay in *T. vesiculosum* conducted using total protein isolated from untransformed tobacco (UN), putative transformant T1, putative transformant T2. BC indicates buffer control. The inhibition zone is indicated by an arrow. Hyphal morphology of *T. vesiculosum* taken from the growing tip of hypha (A) near the disc containing untransformed tobacco protein showing intact hypha (B) near the disc containing transgenic line T2 protein. Arrows indicate distorted hypha (C) and increased hyphal branching (D) near the disc containing transgenic line T2 protein. Arrow indicates hyphal tip abnormalities.

al. 2012). In this study, tobacco was used as a model system to functionally evaluate the potential of *CeChi1* driven by ubiquitin promoter.

In transformation studies, the initial selection of transformed tissues using selection markers is a crucial step to identify transgenic/non-transgenic events. Hygromycin is a common plant selection marker in several vectors. The concentrations of hygromycin used across different plants range from 10 mg/L up to 200 mg/L (Cho & Cosgrove 2000; Rivarola et al. 2009). However, the working concentration varies with cell type, media, growth conditions and cell metabolic rate (Tuncer 2006). In the present study, hygromycin concentration of 25 mg/L was optimized for selection of transgenic lines, which was in agreement with earlier reports (Tuncer 2006; Chaudhry & Rashid 2010; Ee et al. 2014).

The expression of the *CeChi1* gene in transgenic tobacco was quantified using RT-qPCR analysis. The result denoted the stable expression of chimeric *CeChi1* in transgenic plant without silencing phenomena. The study demonstrated that monocot-derived ubiquitin promoter could effectively control transgene expression in dicot plants. Several studies have reported the use of RT-qPCR to analyse the expression of transgene in putative transformants, like transgenic maize expressing *bar* selectable marker gene (Assem & Hassan 2008), transgenic tobacco expressing *Trichoderma harzianum* endochitinase (Saiprasad et al. 2009) and transgenic *Elaeis guineensis* expressing polyhydroxybutyrate gene (Ismail et al. 2010).

In vitro antifungal activity of total protein isolated from untransformed and putative transformant

The fungal inhibition assay was conducted against *T. vesiculosum*, *F. oxysporum* and *R. solani*. A prominent inhibition zone was observed near the disc loaded with total proteins extracted from T2 when compared to untransformed control. No inhibition was observed near the disc loaded with buffer-only (Figs 4–6). Microscopic analysis of the hyphae revealed a clear lysis of the hyphal tips in all tested pathogens when treated with proteins extracted from transgenic line T2. Morphological distortions like increased volume and branching of hyphae was documented in *T. vesiculosum* (Fig. 4). An intact hyphal tip was observed in hyphae exposed to buffer control and proteins isolated from untransformed plant (Figs 4–6). Analysis was also conducted to determine the width and volume of control and treated hyphae. A significant increase in hyphal width and volume was recorded in *T. vesiculosum* treated with leaf protein from T2 when compared to hyphae treated with proteins isolated from untransformed control. No significant variation in hyphal width and volume was documented in *R. solani* and *F. oxysporum* (Fig. 7).

In planta evaluation of CeChi1 in tobacco

The transgenic line T2 and wild-type control plants were challenged with the fungal pathogen *R. solani* to assess level of disease tolerance of transgenic tobacco expressing *CeChi1* gene. The mock-inoculated plants showed no morphological or developmental abnormalities during the period of study (4 weeks). In the con-

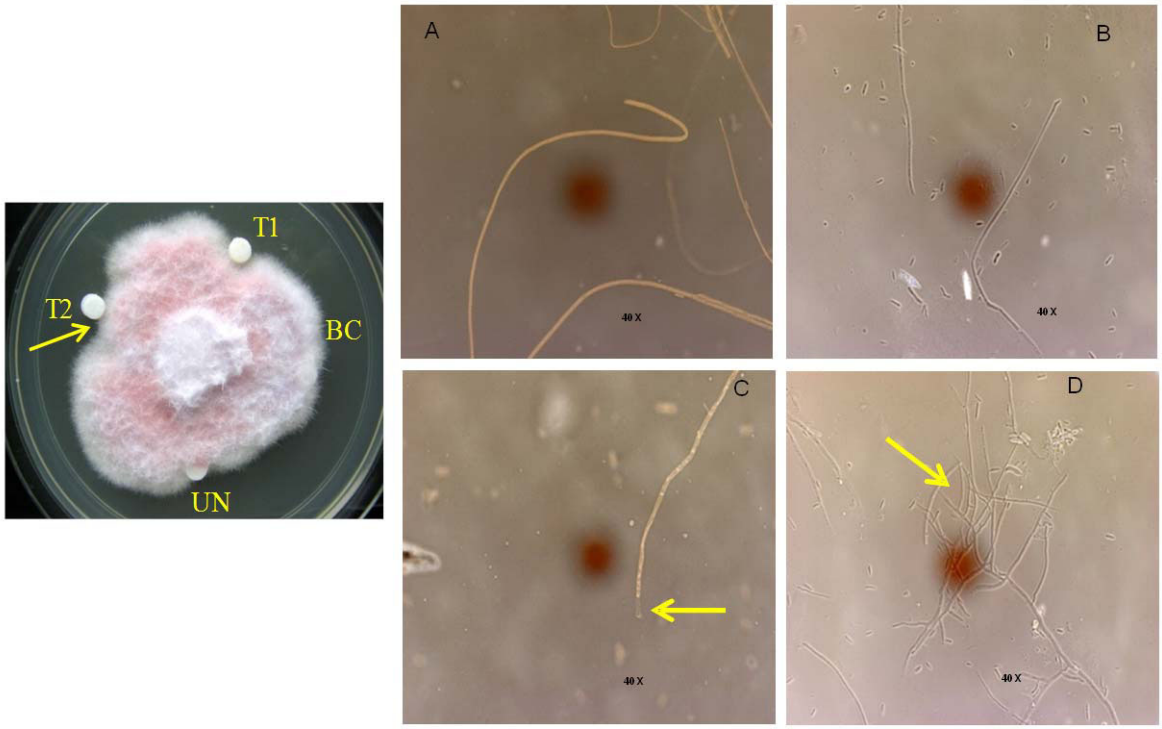

Fig. 5. *In vitro* hyphal morphology studies against *Fusarium oxysporum*. Hyphal extension inhibition assay in *F. oxysporum* conducted using total protein isolated from untransformed tobacco (UN), putative transformant T1, putative transformant T2. BC indicates buffer control. The inhibition zone is indicated by an arrow. Hyphal morphology of *F. oxysporum* taken from the growing tip of hypha (A) near the disc containing untransformed tobacco protein showing intact hypha (B) near the disc containing untransformed tobacco protein showing intact hypha (C) near the disc containing transgenic line T2 protein. Arrows indicate lysed hypha (D) and lysed hyphal tip (D) near the disc containing transgenic line T2 protein. Arrow indicates increased abnormal branching.

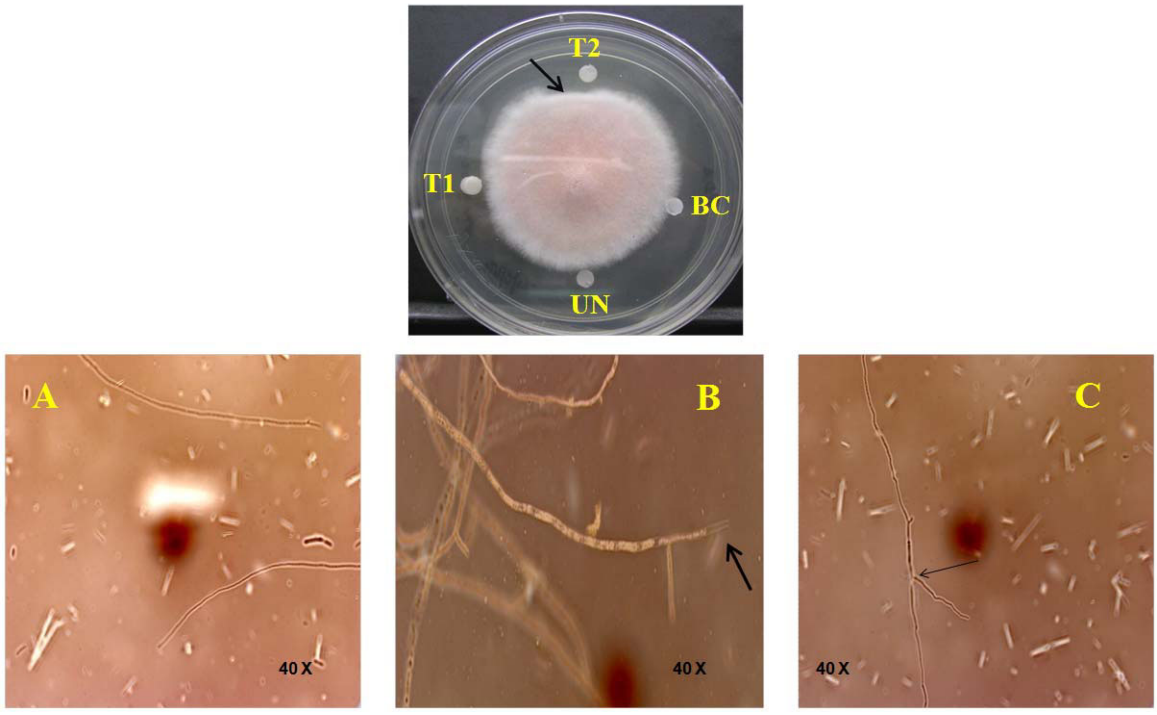

Fig. 6. *In vitro* hyphal morphology studies against *Rhizoctonia solani*. Hyphal extension inhibition assay in *R. solani* conducted using total protein isolated from untransformed tobacco (UN), putative transformant T1, putative transformant T2. BC indicates buffer control. The inhibition zone is indicated by an arrow. Hyphal morphology of *R. solani* taken from the growing tip of hypha (A) near the disc containing untransformed tobacco protein (B) near the disc containing transgenic line T2 protein. Arrows indicate lysed hyphal tip (C) near the disc containing transgenic line T2 protein. Arrow indicates hyphal distortion.

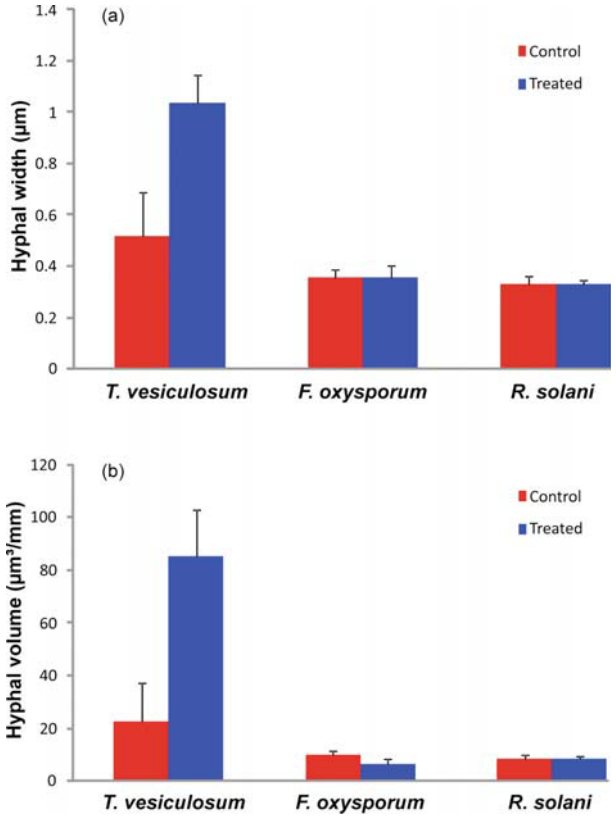

Fig. 7. Variations in hyphal width (a) and volume (b) of pathogens treated with total proteins isolated from untransformed (control) and transformed (T2) plantlets.

trol untransformed plant, symptoms like water-soaked lesions in leaves and stem, yellowing of leaves and severe wilting, which are characteristic of *R. solani* infection, were observed 4-5 days after infection, while no visible symptoms were found in the leaves of transgenic line T2 (Fig. 8).

The antifungal nature of different groups of chitinases are well documented from plants like *Arabidopsis thaliana* (Samac et al. 1990), bean (Awade et al. 1989), tobacco (Sela-Buurlage et al. 1993), winged bean (Esaka and Teramoto 1998), cucumber (Metraux et al. 1989), pea (Chang et al. 1995), soybean (Yeboah et al. 1998), *Vitis vinifera* (Roberts et al. 2002), *Sorghum bicolor* (Mincoff et al. 2006), barley (Kirubakaran & Sakthivel 2007), *Brassica* (Ahmed et al. 2012) and sugarcane (Wang et al. 2014; Su et al. 2015). Class I chitinases have also been reported from carnivorous plants *Ancistrocladus, Dionaea*, *Drosera*, *Nepenthes* and *Triphyophyllum* (Renner & Specht 2012). Heterologous expression of chitinases in transgenic systems has shown enhanced disease resistance in species, such as grapevine, *Theobroma cacao*, tobacco, *Nicotiana benthamiana* and banana (Yamamoto et al. 2000; Dana et al. 2006; Maximova et al. 2006; Xiao et al. 2007; Kovács et al. 2013).

In the present study, the *in vitro* bioassay confirmed the stable expression and functionality of the *CeChi1* transgene. Similar confirmation of transgene ex-

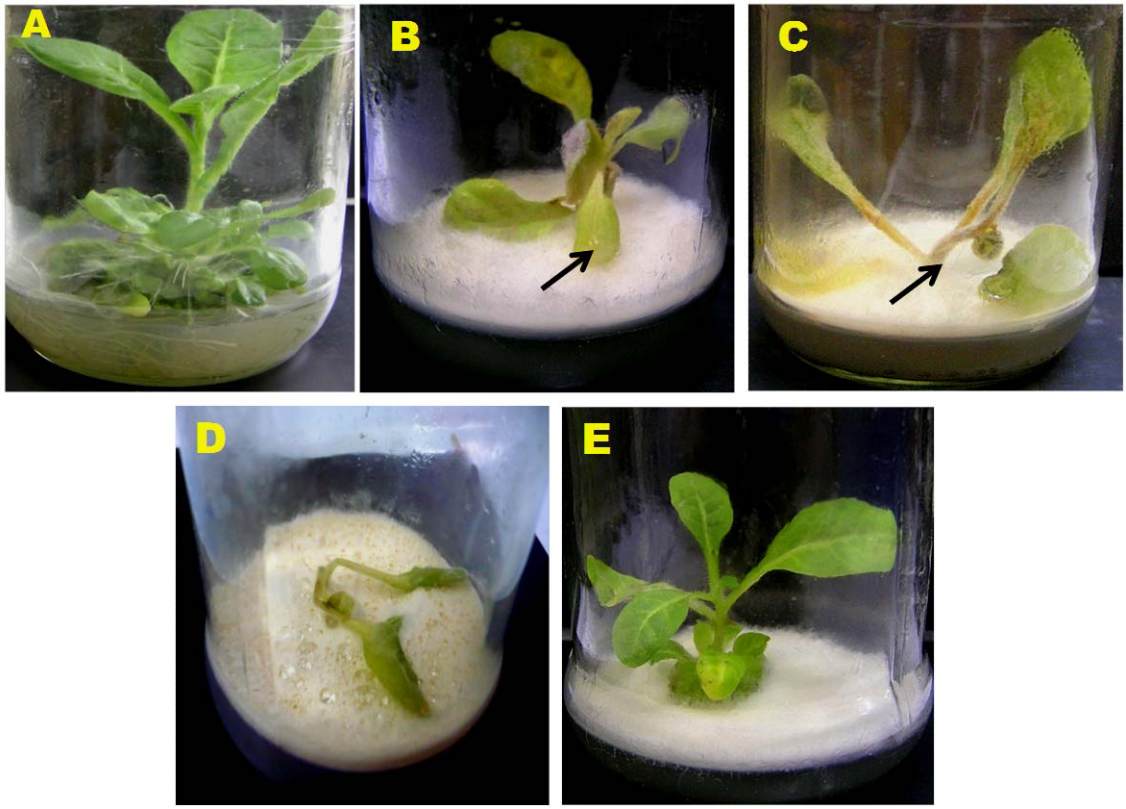

Fig. 8. *In planta* evaluation of transgenic tobacco expressing *CeChi1* against *Rhizoctonia solani*. (A) Mock inoculated. (B) Untransformed control displaying symptoms of yellowing of leaf (after 4 weeks) indicated by an arrow. (C) Untransformed control displaying symptoms of brownish water soaked lesions (after 4 weeks) indicated by an arrow. (D) Untransformed control displaying symptoms of wilting (after 4 weeks). (E) Transgenic line T2 with reduced symptoms.

pression in tobacco was reported by Saiprasad et al. (2009), where the efficacy of total protein extracts of transgenic tobacco expressing *Tricoderma harzianum* endochitinase in inhibiting fungal growth was demonstrated. Leaf extracts from the transgenic tobacco lines expressing a *Saccharomyces cerevisiae* chitinase inhibited *Botrytis cinerea* hyphal growth by up to 70% in a quantitative *in vitro* assay, leading to severe physical damage on the hyphae (Carstens et al. 2003). In another study, chitinase gene from bean was expressed in cotton and total leaf proteins isolated from transgenic cotton inhibited the hyphal extension of *Verticillium dahlia* (Tohidfar et al. 2009).

In planta validation of the *CeChi1* in putative transgenic line was evaluated by challenging the transformed and untransformed plantlets with *R. solani*. *R. solani* is a basidiomycetous soil borne pathogen and earlier studies have shown that the fungus infects tobacco (Broglie et al. 1991; Logemann et al. 1992). The *R. solani*/tobacco system was reported as a suitable system for studying the ectopic expression of genes with antifungal activity (Jach et al. 1995). In the present study, the *in planta* analysis revealed that the transgenic tobacco line exhibited an increased tolerance to the pathogen infection when compared to untransformed line. The results corroborate and extend the observation of Broglie et al. (1991), that constitutive expression of plant chitinase can enhance the resistance to *R. solani*.

Overexpression of chitinase genes from different origins in transgenic system are known to enhance resistance to fungal pathogens and other environmental stresses (Jach et al. 1995; Hong & Hwang 2006). Several reports on antifungal effect of chitinases expressed in transgenic system against *R. solani* have been reported from indica rice (Sridevi et al 2003), potato (M'hamdi et al. 2012), cotton (Emani et al. 2003), *Agrostis palustris* (Chai et al. 2002) and forage legume *Stylosanthes guianensis* (Kelemu et al. 2005). Similarly, in the present study the *CeChi1* isolated from *C. equisetifolia* was demonstrated to enhance the tolerance of transgenic tobacco to *R. solani*.

This study revealed that the class I chitinase isolated from *C. equisetifolia* can act as a potential gene resource in future transformation programs for incorporating disease tolerance caused by fungal pathogens.

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