Research Article

Antifungal activity of chitinase II against *Colletotrichum falcatum* Went. causing red rot disease in transgenic sugarcane

Muhammad TARIQ[1], Anwar KHAN[2], Bushra TABASSUM[1,*], Nida TOUFIQ[1],
Muhammad Umar BHATTI[1], Saman RIAZ[1], Idrees Ahmad NASIR[1], Tayyab HUSNAIN[1]
[1]Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan
[1]Department of Genetics, Hazara University, Mansehra, Khyber Pakhtunkhwa, Pakistan

Abstract: We evaluated transgenic lines of sugarcane modified with the barley chitinase class-II gene to create resistance against the red rot causative agent *Colletotrichum falcatum* Went. Local sugarcane cultivar SP93 was transformed with a 690-bp coding sequence of the chitinase-II gene under the influence of a polyubiquitin promoter. Transgenic sugarcane lines (T_0) overexpressing the chitinase gene were obtained through a particle bombardment method with 13.3% transformation efficiency. Four transgenic sugarcane lines, SCT–03, SCT–05, SCT–15, and SCT–20, were tested for resistance against red rot by in vitro antifungal assays. Crude protein extracts from transgenic sugarcane plants SCT–03, SCT–05, SCT–15, and SCT–20 inhibited the mycelial growth of *C. falcatum* by 49%, 40%, 56%, and 52%, respectively, in a quantitative in vitro assay. Our findings revealed that two transgenic lines, SCT–15 and SCT–20, exhibited the highest endochitinase activity of 0.72 and 0.58 U/mL, respectively. Furthermore, transgenic lines SCT–15 and SCT–20 exhibited strong resistance against inoculated *C. falcatum* in an in vitro bioassay, as they remained healthy and green in comparison with the control sugarcane plants, which turned yellow and eventually died 3 weeks after infection. The mRNA expression of the transgene in the *C. falcatum*-inoculated transgenic sugarcane lines increased gradually compared to the control plant. The mRNA expression was the highest at 72 h in both transgenic lines and remained almost stable in the subsequent hours.

Key words: Red rot tolerant sugarcane, antifungal gene, *Colletotrichum falcatum* Went., sugarcane transformation, chitinase gene, quantitative in vitro fungal bioassay, chitinase activity, transgene mRNA expression

1. Introduction

Fungal diseases are responsible for considerable yield losses in sugarcane crop. About 160 fungi have been reported to infect sugarcane, while seven diseases with unknown etiologies have been recognized and documented (Rott, 2000). Fungal diseases reduce cane yield by up to 31% (Jayashree et al., 2010). Among them, the most common disease is red rot, which is also known as a "cancer" of sugarcane (Khan et al., 2011), and is caused by the fungus *Colletotrichum falcatum* Went. Red rot severely affects the quantity and quality of the cane, with about a 29% reduction in cane weight and 30% less sugar recovery (Hussnain and Afghan, 2006).

Red rot is one of the major, oldest, broadly distributed, and documented diseases of sugarcane in many countries, including Pakistan, the United States, Bangladesh, Taiwan, and Thailand (Viswanathan and Samiyappan, 2002; Viswanathan, 2012). It infects the sugar cane stalk at both the initial and mature stages of growth, causing discoloration, while the pathogen-produced invertase enzyme causes sucrose inversion in addition to drying of the cane stalks. Thus, overall, the vegetative growth of the plant halts (Sharma and Tamta, 2015). Various methods are adopted for red rot disease management, including chemical, biological, breeding, and tissue culture methods. All of these have certain limitations and disadvantages. For instance, chemical methods increase the cost of production and contaminate the environment as well, while biological control applied through plant growth-promoting bacteria (Malathi and Viswanathan, 2013) does not show consistent results in the field. Similarly, in tissue culture, the selection of fungus-resistant cells (Mohanraj et al., 2003; Sengar et al., 2009) is most often associated with somaclonal variations. Red rot-resistant varieties can be produced by breeding methods (Agnihotri, 1996), but breeding is laborious and time-consuming. To overcome all these limitations, genetic modification is the best available option for management of red rot disease in sugarcane crops. Specific genes can be introduced into the genome of sugarcane to create resistance against a particular pathogen (Enríquez-Obregón et al., 1998).

* Correspondence: bushra.cemb@gmail.com

Chitinases are lytic enzymes that act on chitin, which is the major constituent of the cell wall of the majority of fungi. Chitinases are proteins related to pathogenesis and are involved in the plant defense response upon pathogen infection (Shin et al., 2008; Su et al., 2015). The hydrolytic action of chitinase results in the degradation of the fungal cell wall.

We manipulated the sugarcane genome with a barley-derived endochitinase II gene for effective control of red rot disease. Studies were carried out to assess the antifungal properties of the chitinase II gene isolated from barley. The results of these studies are reported in this manuscript.

2. Materials and methods

2.1. Construct details
The chitinase II (CEMB–chiII) gene, with accession no. KC899774.1, was kindly provided by the Seed Biotechnology Lab, Centre of Excellence in Molecular Biology (CEMB), Lahore, Pakistan. The ~690-bp chitinase gene was cloned at multiple cloning sites (MCSs) of a binary vector, pCAMBIA1301, downstream of a polyubiquitin promoter. The gene was initially amplified with primers to modify the transgene fragment with the desired restriction site to facilitate directional cloning. The chitinase II gene was amplified with the forward primer *BamHI* restriction site 5′-GACGGATCCGTCATCACGCAATCGGTGTA-3′ and reverse primer *KpnI* restriction site 5′-GCGGGTACCGAAGTTTCGCTGGGTGTAGC-3′. At the MCS of pCAMBIA1301, the polyubiquitin promoter was cloned at the *HindIII* and *BamHI* restriction sites followed by a transgene that was cloned at the *BamHI* and *KpnI* sites, while the NOS terminator was cloned at the *KpnI* site to construct pUbi–chiII (Figure 1). The ligated products were moved into chemically competent *E. coli* DH5α cells through a heat-shock method as adopted from Froger and Hall (2007). Positive clones were selected after restriction digestion and subsequently through sequencing (Figure S1).

2.2. Plant material
Sugarcane (*Saccharum officinarum* L.) cultivar S2006SP-93 was obtained from the Sugarcane Research Institute of Faisalabad, Pakistan. The plants were grown in growth chambers with a 16-h light period, with 600 mE m^{-2} s^{-1} light intensity and a temperature regime of 20 °C : 18 °C (light : dark). Fresh light green leaf tissues were taken from the inner whorl of the sugarcane spike and were sterilized by immersion in a solution containing 0.1% mercuric chloride and 0.1% Tween-20 for 10–15 min and were later rinsed in sterile distilled water. Furthermore, a piece of leaf of 1–2 mm was used as an explant to induce friable embryogenic calli. The leaf disks were placed onto S1 medium [MS basal medium (Murashige and Skoog, 1962) supplemented with 1.0 g/L casein hydrolysate, 2 mg/L 2,4-D, 3% sucrose, and 2.23 g/L Phytagel; pH 5.7] plates and were placed in the dark at 25 °C for 5–7 days to initiate calli. Green nodular embryogenic calli were transferred to CM medium [S1 medium with 6 g/L Phytagel] 4–16 h prior to genetic transformation.

2.3. Nuclear transformation
The genetic transformation of sugarcane cultivar S2006SP-93 was done through particle bombardment using a homemade biolistic gun [developed by CEMB, in collaboration with the Pakistan Atomic Energy Commission]. A total of 5 μg of the construct pUbi-chiII was coated with 60 μL of tungsten particles. Furthermore, 50 μL of 2.5 M sterilized CaCl$_2$ solution was added, followed by 20 μL of 0.1 M spermidine. The whole reaction mixture was vortexed thoroughly. In total, 40 μL of the mixture that comprised DNA-coated tungsten particles was loaded onto the center of the filter assembly inside the locally developed biolistic gene gun. Bombardment of the DNA-coated tungsten particles was carried out under a partial vacuum of 25 mmHg and 1500 psi pressure of helium gas. Sixteen hours after the bombardment, the calli were shifted to the regeneration medium (S2) adopted from Nasir et al. (2014). For the controls, the empty pCAMBIA1301
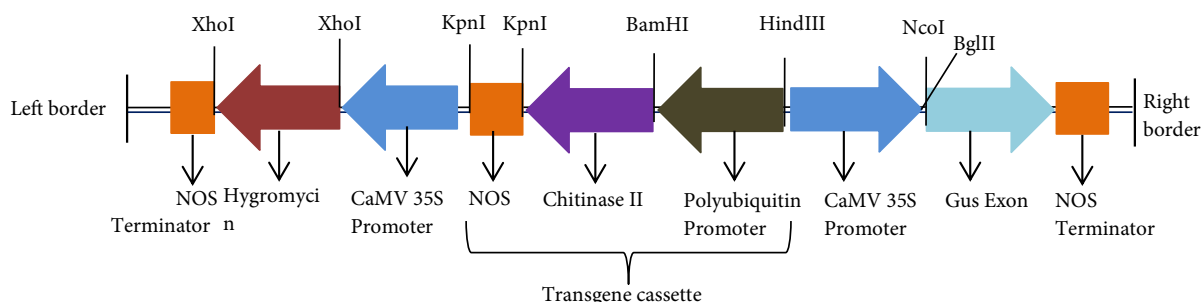

Figure 1. Diagrammatic outline of the binary construct (pUbi–ChiII) that was used to genetically transform sugarcane cultivar SP93. The transgene, the chitinase II gene, was cloned in the MCS of the pCAMBIA1301 vector at the *BamHI* and *KpnI* sites while it was driven by the polyubiquitin promoter that was cloned upstream of the transgene with the *HindIII* and *BamHI* sites. The gene was terminated with an NOS terminator that was cloned at the *KpnI* site.

vector, without transgene, was used to bombard sugarcane calli and proceeded as described above. After 7 days of transformation, the proliferating calli pieces were shifted onto S2 medium plates additionally supplemented with 150 mg/L kanamycin as described by Mustafa and Khan (2012). The sensitivity of calli to the antibiotic and the survival rate of the transformed cells along with the regeneration response was evaluated.

The complete regenerated plantlets were subcultured onto MS medium in combination with 3% sucrose as a carbon source with pH 5.7–5.8 in glass tubes for about 2–3 weeks for the development of shoots and roots. The sugarcane plants that developed from the empty vector were designated as T_0 plants.

2.4. PCR screening
The potential transgenic sugarcane plants were analyzed by transgene amplification with a specific primer pair. DNA extraction of the transformed sugarcane plants was done with a genomic DNA purification kit (Thermo Scientific), and the isolated DNA was used as a template to amplify the transgene for screening potential transgenic sugarcane plants. A primer pair specific for the chitinase II gene was used to amplify the transgene. The PCR products were resolved on a 1% agarose gel and were stained with ethidium bromide.

2.5. Confirmation of the transgene integration
A Southern blot was performed to verify the transgene integration in the genome of the transformed sugar cane plants. A chitinase gene fragment of ~690 bp was labeled with digoxigenin (DIG) using a DIG DNA Labeling and Detection Kit (Roche) as per the instructions. Approximately 20 μg of genomic DNA of regenerated sugarcane plants was digested with the restriction enzymes *BamHI* and *KpnI*. The digested fragments were resolved on 0.8% agarose gel and were blotted onto Hybond-N+ nylon membranes (Amersham, USA). The membranes were probed with DIG-labeled coding region fragments of the barley chitinase II gene. The hybridization was carried out at 65 °C for 16 h followed by detection through an enzymatic reaction where BCIP/NBT tablets were used as a substrate against the alkaline phosphatase enzyme.

The transformation efficiency was calculated on the basis of the preliminary molecular analysis including the PCR and Southern blot.

2.6. Fungal inhibition assay
The promoter-driven constitutive expression of the recombinant chitinase protein in the transgenic sugarcane plants was revealed by an in vitro fungal inhibition assay and an endochitinase activity assay. Total crude protein extract of transgenic sugarcane plants was used for the in vitro fungal inhibition assay adopted from Mondal et al. (2003). One gram of fresh leaf sample from the transgenic sugarcane plants was collected, ground in liquid nitrogen,

and resuspended in 500 μL of the protein extraction buffer. The samples were centrifuged at 13,200 × *g* for 20–25 min at 4 °C. The supernatant corresponded to the crude protein extract and was used directly in the inhibition assay. A spore suspension of 20 μL (2×10^6 spores/mL) of *C. falcatum* was added into the wells made in the center of potato dextrose agar (Oxoid) plates and was incubated at 28 °C overnight. Later, 50 μg of the extracted crude protein from the transgenic sugarcane plants was added to the wells previously inoculated with *C. falcatum* and the incubation continued at 25 °C for 3 days. The negative control was prepared by adding the same concentration of fungal inoculum (20 μL of spore suspension of 2×10^6 spores/mL) to the well, and 50 μg of the crude protein from the T_0 plant was applied.

The radial growth of the fungus was recorded daily for up to 3 days. The percent inhibition of *C. falcatum* hyphal growth was calculated as the ratio of the diameter of the fungal colony in the treatment to the diameter of the fungal colony in the control sample multiplied by 100 (Plascencia-Jatomea et al., 2007).

2.7. Endochitinase activity assay
The presence of recombinant chitinase protein in the transgenic sugarcane plants was revealed by an endochitinase activity assay (Sigma). The kit was based on a fluorometric assay, which involves the enzymatic hydrolysis of the substrate, chitin, and the absorbance of the released substrate was measured at 405 nm. The total crude protein extract from the transgenic sugarcane plants was isolated and quantified by the Bradford assay (Bradford, 1976). Of the 200 μL of total crude protein, 10 μL was placed in a 96-well ELISA plate, and 90 μL of the substrate 4-nitrophenyl β-D-N, N',N''-triacetylchitotriose was added. The plate was incubated at 37 °C for 30 min followed by an absorption measurement at 405 nm. For the negative control, the crude protein extract from the T_0 sugarcane plants was used for the endochitinase activity assay, while *Trichoderma* purified chitinase, provided in the kit, was used as a positive control. The amount of endochitinase was calculated using the procedure described by Bergmeyer et al. (1974):

U/mL = (A_{405}sample × A_{405}blank) × 0.05 × 0.3 × DF / A_{405}standard × time × V_{enz},

where A_{405}sample is the absorbance of the sample at 405 nm; A_{405}blank is the absorbance of the blank at 405 nm; 0.05 represents mmol/mL p-nitrophenol in the standard solution; 0.3 represents the final volume of the 96-well plate reaction after addition of the stop solution (mL); DF is dilution factor, the fold dilution of the original chitinase enzyme or biological solution to prepare sample for the test; A_{405}standard is the absorbance of the standard solution at 405 nm; time is in minutes; and V_{enz} is the volume of the sample (mL).

2.8. Antifungal bioassay on transgenic sugarcane plants

The resistance of the transgenic sugarcane plants against *C. falcatum* was assessed by antifungal bioassays. Transgenic sugarcane plants exhibiting a significantly high fungal inhibition in the in vitro assays were selected. The method was adopted from Yevtushenko et al. (2005) with slight modifications. Thirty-day-old transgenic sugarcane plants and control plants (T_0) were assessed in this assay. For the bioassay of the whole plants, the transgenic and control plants were subcultured in test jars. After 10 days, a 0.5-cm² PDA agar block of *C. falcatum* with good mycelial growth was placed in the jar. The inoculated plants were placed in the light at 25 ± 2 °C under a 16/8-h photoperiod and were observed for any morphological symptoms particular to *C. falcatum* and survival for up to 15 days.

2.9. Differential mRNA expression of the chitinase gene in the transgenic sugarcane plants

Transgenic sugarcane plant exhibiting high antifungal potential in the in vitro bioassay was selected for mRNA expression. The transgenic sugarcane plant grown in MS media was inoculated with 1 mL of the spore suspension of *C. falcatum*, corresponding to 10^4 spores. For mRNA expression of the chitinase gene, the samples were collected at 24 h, 48 h, 72 h, 96 h, and 120 h after fungal inoculation. Total RNA was isolated from the leaf samples at 48 h after fungal inoculation by using TRIzol reagent (Invitrogen) as per the manual. Then 1 μg of total RNA was used to synthesize the cDNA using a cDNA synthesis kit (Thermo Scientific) as per the instructions. This cDNA was used as the template in the subsequent semiquantitative real-time PCR assay with forward (5'-AGCCCAGGGTAACAAACCAT-3') and reverse (5'-CCAAGCATACCGCAATACCT-3') primers. Real-time PCR was performed on a quantitative thermocycler with Piko Real 3.1 software (Thermo Scientific) using

SYBR Green qPCR 2X Master Mix (Thermo Scientific). The reaction profile was denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s. Beta-actin was used as the internal control for normalization, which was amplified with the forward primer 5'-ATGTTCCCGGGTATTGCTGACAG-3' and the reverse primer 5'-CTGCCTTTGCAATCCACATCTGCT-3'. Relative gene expression analysis was done by using the Ct values of the different samples and the standard deviation was calculated. Each real-time PCR assay was performed in triplicate.

3. Results

3.1. Transformation and molecular characterization of the transgenic sugarcane plants

The main aim of the research was to develop transgenic lines of sugarcane tolerant against the red rot causative agent, *C. falcatum*. The constructed binary vector, pUbi–chiII, harboring the ubiquitin promoter, the chitinase II gene, and NOS terminator, was propagated in *E. coli* cells and was confirmed at each step of cloning through a restriction digestion. Figure 2 represents the different steps of the pUbi–chiII construct synthesis. The successful release of a specific insert from the total vector represented positive cloning. Furthermore, a high concentration of the pUbi–chiII vector was obtained with a maxi prep that was used in the bombardment experiment later.

In total, 30 regenerated plantlets were obtained after transformation that survived the kanamycin selection, while 15 T_0 plantlets were obtained after transformation with the empty pCAMBIA1301 vector, and these were treated as control plants.

While screening the transformed sugarcane plants, out of the 30 regenerated sugarcane plantlets transformed with the pUbi–chiII construct, only 4 plantlets (SCT–03, SCT–
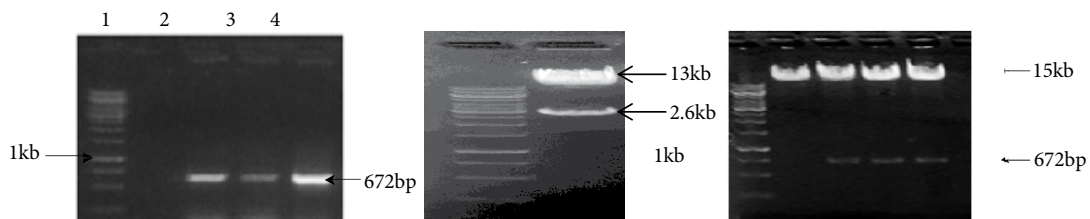

Figure 2. Sequential steps involved in the construction of plant binary vector pUbi–ChiII. A) Amplification of the chitinase II gene with gene specific primers. Lane 1: 1-kb DNA ladder; lane 2: negative control; lanes 3–5: sample DNA. b) Restriction digestion of the pUbi–ChiII construct to verify the ligation of the polyubiquitin promoter. The appearance of two distinct sharp and defined bands at a specific size verifies positive cloning. The gel fragment at ~13 kb depicts the vector, while the ~2.6-kb fragment depicts the polyubiquitin fragment. Lane 1: 1-kb DNA ladder; lane 2: sample DNA of pUbi–ChiII digested with the *HindIII* and *BamHI* restriction enzymes. c) Restriction digestion of the pUbi–ChiII construct to verify the ligation of the chitinase II gene. The appearance of two distinct sharp bands corresponding to ~15 kb depicts the vector, while the ~672-bp fragment depicts the chitinase II gene. Lane 1: 1-kb DNA ladder; lane 2: pUbi–Chi vector (T_0) without transgene; lane 3: sample DNA of pUbi–ChiII digested with the *BamHI* and *KpnI* restriction enzymes.

05, SCT–15, and SCT–20) were positive when analyzed by PCR with gene-specific primers as shown in Figure 3a. A sharp and clear amplification at ~672 bp was observed in four samples, while in the T_0 plantlets no amplification of the transgene was observed. The regenerated sugarcane plants transformed with the pUbi–chiII construct that were positive by PCR were subjected to Southern blotting, whereby all the PCR positive plants, including the SCT–03, SCT–05, SCT–15, and SCT–20 plants, were positive upon hybridization with the chitinase II gene probe when spotted onto a nylon membrane (Figure 3b). The transformation efficiency was estimated to be 14% to 28% in duplicate, as shown in the Table.

3.2. Expression of the chitinase II gene in sugarcane
To assess the functionality of the protein in the transgenic sugarcane lines, an in vitro fungal inhibition assay was performed with the crude protein extracts from the transgenic sugarcane plants. Transgenic sugarcane plant SCT–15 exhibited the maximum inhibition of red rot causative fungus *C. falcatum*, which was 56%, while the transgenic sugarcane plant SCT–03 inhibited *C. falcatum* growth by 49%. In addition, the crude protein of SCT–05 inhibited fungal growth by up to 40%, and transgenic sugarcane plant SCT–20 inhibited *C. falcatum* growth by 52% (Figure 4). These were in comparison with the control sample (T_0), where no inhibition of subjected fungal growth was revealed.

Similarly, the chitinase activity of the total transgenic plant protein extract was quantified by fluorescence assays using the specific substrate 4-methylumbelliferyl-b-D-N,N',N'',N'''-tetraacetylchitotetraoside [4-MU-(GINAc)4] (Figure 5). All four transgenic sugarcane plants, SCT–03, SCT–05, SCT–15, and SCT–20, were positive for the endochitinase activity assay. However, the enzyme quantity varied among the lines. Figure 5 shows that in

the transgenic sugarcane plant SCT–03, 0.64 U/mL of the enzyme endochitinase was present, while SCT–05 had 0.6 U/mL and SCT–20 had 0.58 U/mL, and SCT–15 showed the maximum units of endochitinase at 0.72 U/mL. The positive control provided in the kit gave 5.67 U/mL of endochitinase. These results showed that the protein produced by the transgene is functional and hence exhibits chitinase activity.

3.3. Overproduction of chitinase II promotes disease resistance of transgenic sugarcane to red rot pathogens
On the basis of the in vitro fungal inhibition assay and the endochitinase activity assay, two transgenic sugarcane lines, SCT–15 and SCT–20, were selected for the bioassay. Transgenic sugarcane lines SCT–15 and SCT–20 and the control T_0 plants were infected with *C. falcatum*. After 2 weeks, the control sugarcane plant turned brown and weak, and it eventually died in the third week (Figure 6), while transgenic sugarcane plant lines SCT–15 and SCT–20 remained green and healthy until the 2nd and 3rd weeks after *C. falcatum* infection. This result showed that the overproduction of chitinase rendered the plants tolerant against red rot infection. Although the inoculated fungi had overgrowth in the jars where the plants were present, as shown in Figure 6, fungal mycelia were seen on the stem of the subjected plant but at the third week of infection, and the control plant turned brown and died eventually while the transgenic plant remained stable. It is noteworthy that the subjected transgenic sugarcane plant lines SCT–15 and SCT–20 showed maximum enzyme activity in the endochitinase activity assays. Hence, transgenic sugarcane lines SCT–15 and SCT–20 not only survived even after heavy infection of red rot but also remained healthy and green.

The effect of inoculated fungal stress on the mRNA expression of the transgene in transgenic sugarcane plants
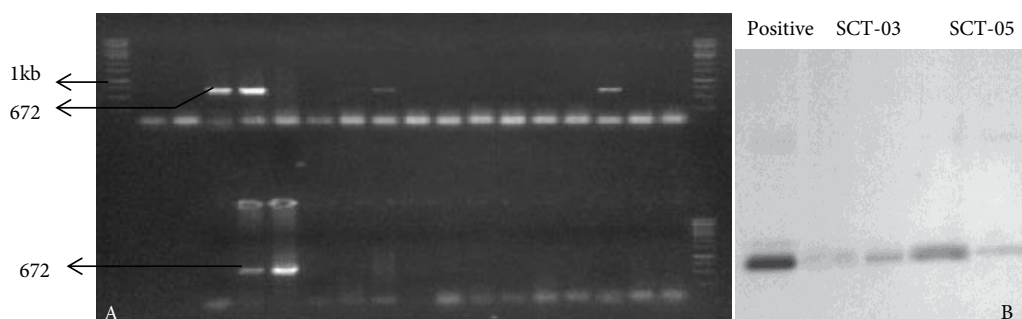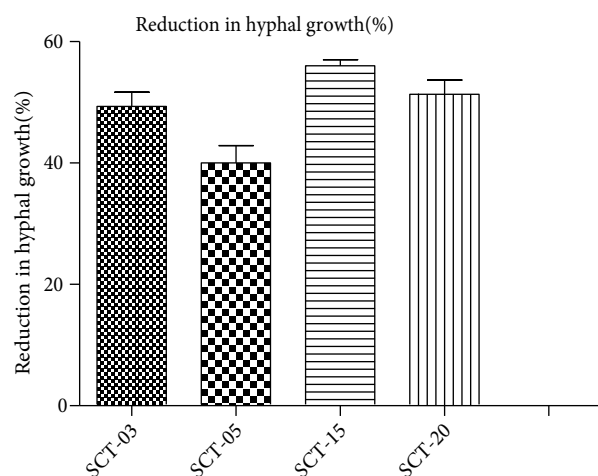

Figure 3. Molecular analyses to screen the potential transformed sugarcane plants. A) Amplification of the transgene from the transformed sugarcane plants using genomic DNA as the template. A sharp amplified fragment of ~672 bp was clearly seen in a few of the plant samples, while for the others, no product was amplified, depicting untransformed sugarcane plant samples. b) Southern blot was used to reveal that the chitinase II gene was integrated into the genomic DNA of the transformed sugarcane plants. The chitinase II gene fragment was DIG-labeled and used as a probe. "Positive" refers to the plasmid DNA, pUbi–ChiII.

Table. Transformation efficiency of sugarcane cultivar SP-93 when the particle bombardment method through a gene gun was adopted.

Sugarcane variety	Construct transformed	Total no. of callus pieces transformed	No. of plants regenerated on antibiotic selection	PCR-positive plants	Southern-positive plants	Transformation efficiency (%)
S2006SP-93	pUbi-ChiII	32	30	04	04	13.3
	pUbi (T0)	24	15	06	-	40

Figure 4. Graphical representation of the % inhibition of the growth of *C. falcatum* using the crude protein extract from the transgenic sugarcane lines. The value obtained from the control plant was deducted to reveal the percentage reduction. The y-axis represents the % inhibition, while the x-axis represents the individual transgenic sugarcane plants.
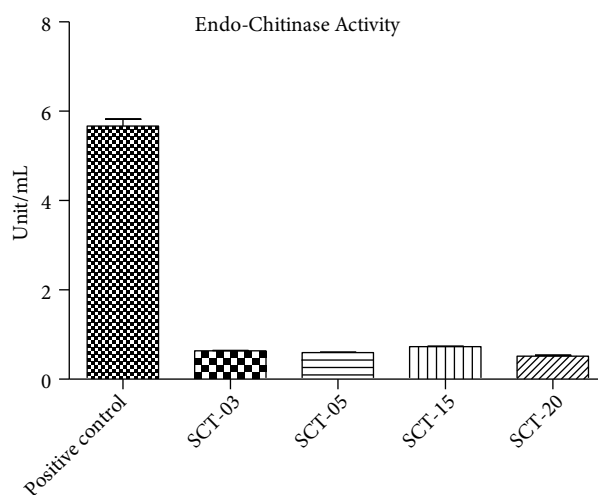
Figure 5. Graphical representation of the chitinase activity assay of the transgenic sugarcane plants. The y-axis represents the units of chitinase enzyme to hydrolyze the substrate, while the x-axis represents the sugarcane plant used.

SCT–15 and SCT–20 was revealed by real-time PCR. Upon infection of *C. falcatum* in the transgenic sugarcane plant SCT–15, the mRNA expression of the transgene initially rapidly reduced to 0.69 at 24 h after infection. However, at 48 h and at later stages, the transgene expression increased and remained almost stable (Figure 7). The mRNA expression at 48 h was 1.4. At 72 h, the expression level was 3.37, and at 96 h, the expression was 2.87, while at 120 h, the expression was 3.13 (Figure 7). In contrast, in transgenic sugarcane line SCT–20, the mRNA expression of the transgene was initially reduced to 0.5 at 24 h, but in subsequent hours, the expression increased gradually and was maximum at 120 h. At 48 h after red rot infection, the expression revealed was 1; at 72 h the expression was 1.7; at 96 h it was 1.9; and at 120 h the mRNA expression was 2.2 (Figure 7).

Thus, it can be concluded that the chitinase activity detected in the transgenic sugarcane is a consequence of chitinase overexpression and responds to its own lytic activity. Additionally, upon fungal stress, the expression of the transgene was initially reduced due to stress, but with

time an increase in expression was observed. Conclusively, transgenic sugarcane lines SCT–15 and SCT–20 have the required tolerance to protect themselves from heavy infection of red rot, and this tolerance is attributed to the high expression of the chitinase gene.

4. Discussion

The main objective of this study was to develop red rot-tolerant transgenic sugarcane by integrating an antifungal gene derived from barley. Red rot caused by *C. falcatum* exhibits enormous variation in pathogenicity on sugarcane (Viswanathan, 2017). Plant chitinases, when transformed in plants, have been reported to confer resistance against fungal disease (He et al., 2008; Iqbal et al., 2012; Jabeen et al., 2015). Barley chitinase had previously been used for its ability to develop fungal resistant crops (Kirubakaran and Sakthivel, 2007; Khan et al., 2017). Generally, the transgene expression in the host plant depends upon the promoter being used. We employed a maize polyubiquitin promoter to express the chitinase gene in sugarcane. This promoter has shown comparatively high expression in monocot plants when compared with the CaMV35S promoter as
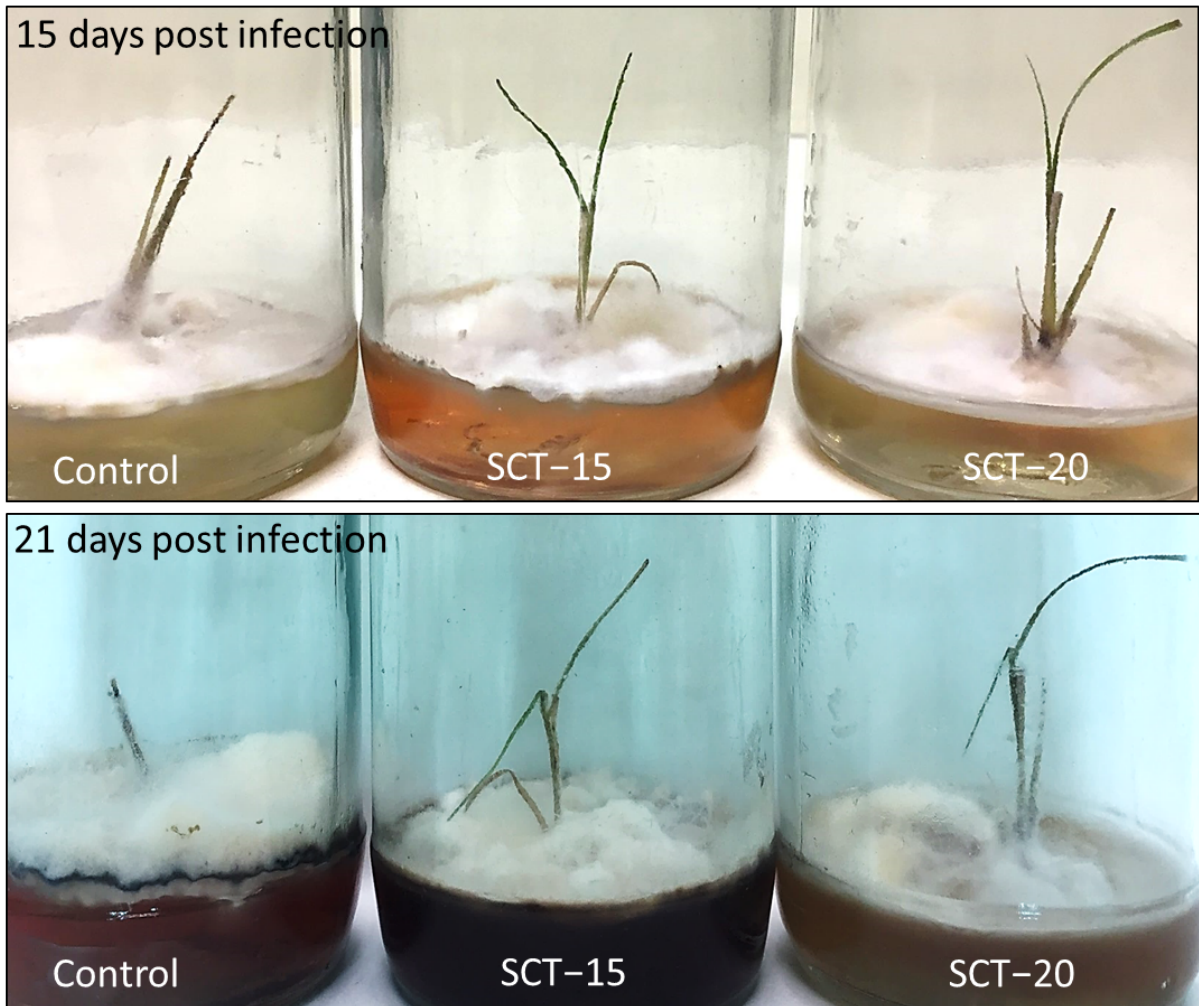
Figure 6. In vitro bioassay of the transgenic sugarcane lines inoculated with *C. falcatum* to evaluate their antifungal potential: the control transgenic sugarcane plant (T_0) at 2 and 3 weeks after infection with *C. falcatum*, the transgenic sugarcane line (SCT–15) 2 and 3 weeks after inoculation with *C. falcatum*, and the transgenic sugarcane line (SCT–20) 2 and 3 weeks after inoculation with *C. falcatum*.

documented by Joung and Kamo (2006). The transgene was moved into the plant by bombardment through a gene gun. Particle bombardment in sugarcane is efficient and rapid and more than one gene can be transformed (Kaur et al., 2007) with 10%–35% transformation efficiency (Wang et al., 1997). In plant transformation, the antibiotics employed for the selection of the transformants differ in their stringency, which depends on their mode of action. Kanamycin has proven effective at a concentration of 150 mg/L in the selection of putative transgenic sugarcane plants as the nontransformed cells die upon selection and only the cells with the foreign construct survive.

Foreign DNA, when introduced into the plant, integrates at random, nonhomologous sites in the genome, where some integrations may occur in transcriptionally active chromatin regions while some others may be located in condensed, transcriptionally inert chromatin regions and thus define reduced or variable expression in the transgenic plants (De Alba et al., 2013). As in our findings, the transgene expression varied among all four transgenic lines of a single plant cultivar. The position of the transgene in the host genome is also associated with the transgene expression levels (Tang et al., 2003).

For a transgene to be effective in protecting a plant against a particular pathogen, the concentration of the recombinant protein must be sufficiently high. Transgenic sugarcane lines SCT–15 and SCT–20 possessed the highest units of chitinase enzyme among the four tested transgenic lines and, in parallel, inhibited the mycelial growth of *C. falcatum* by up to 56% and 52%, respectively, in the in vitro fungal inhibition assay. Our findings are in agreement with Mondal et al. (2003), who generated transgenic plants of
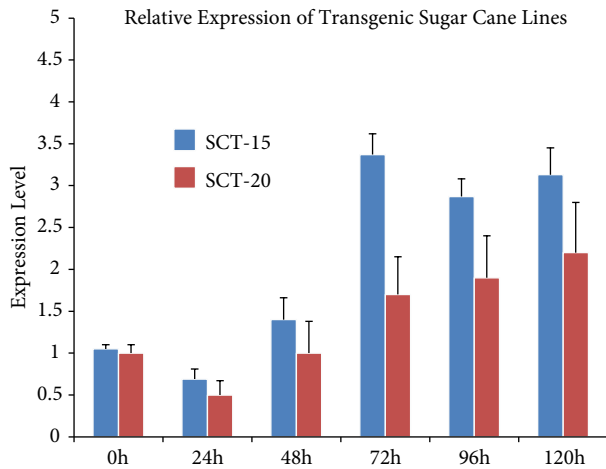
Figure 7. Relative quantification of the chitinase gene after *C. falcatum* infection at various time points to detect the mRNA expression of the chitinase gene in transgenic sugarcane lines SCT–15 and SCT–20 that were inoculated with *C. falcatum*.

Brassica juncea with a chitinase gene insertion. The crude protein extract of transgenic *B. juncea* showed 12% to 56% inhibition of the different tested pathogenic fungi.

To reveal the mRNA expression of the transgene, relative quantification is routinely used in comparison with control and untreated samples. From our findings, upon infection with red rot, the mRNA expression of the transgene rapidly decreased in both tested transgenic sugarcane lines (SCT–15 and SCT–20) but gradually increased in the subsequent hours and remained stable after 72 h; hence, we concluded that chitinase responds to the stress gained by the host plant after it is inoculated with *C. falcatum*. This inoculation of *C. falcatum* induces stress in the subjected transgenic sugarcane plant, which corresponds with reduced mRNA expression of the

transgene. However, the gradual increase in the mRNA expression of the transgene in the subsequent hours of the fungal inoculation documented the tolerance of the transgenic lines against the subjected fungus. This tolerance was attributed to the overexpression of the chitinase gene, which has also been reported as an abiotic stress-tolerant gene (Su et al., 2014).

Several attempts, including the use of pathogenesis-related proteins, have been made to overcome pathogenic fungi in crop plants (Souza et al., 2017). Khan et al. (2016) reported the development of *Rhizoctonia solani*-resistant potato plants through the genetic transformation of the chitinase II gene. Similarly, He et al. (2008) reported *Colocasia esculenta* plants transformed with the rice chitinase gene, and upon infection with fungus, the nontransgenic plants died after 3 to 5 days, while the transgenic plants survived for 1 week after infection. In a similar study, Shin et al. (2008) expressed the barley chitinase II gene in wheat. Their study revealed that the transgenic wheat showed resistance against *Fusarium graminearum* under controlled and field conditions. Recently, Nayyar et al. (2017) expressed β-1,3-glucanase gene from *Trichoderma* spp. to create resistance in sugarcane against *C. falcatum* Went. They revealed that transgene expression in the first clonal generation raised from T_0 exhibited 4.4-fold higher expression.

In conclusion, our findings support a strong correlation between the levels of the transgene transcripts and endochitinase activity, and additionally, the results of the in planta fungal inhibition assays in the transgenic lines confirmed that the inhibitions observed were directly linked to the presence of the heterologously overexpressed barley chitinase protein. These promising results indicate that the barley-derived chitinase gene is an excellent candidate that can be employed for the generation of fungus tolerant crop plants.

References

Agnihotri V (1996). Current sugarcane disease scenario and management strategies. Indian Phytopathol 49: 109-126.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Bergmeyer HU, Gawehn K, Grassl M (1974). Enzymes as biochemical reagents. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis, Volume 1, 2nd ed. New York, NY, USA: Academic Press, Inc., 1974, pp. 495-496.

De Alba AEM, Elvira-Matelot E, Vaucheret H (2013). Gene silencing in plants: a diversity of pathways. Biochim Biophys Acta Gene Regul Mech 1829: 1300-1308.

Enriquez-Obregon GA, Vazquez-Padrón RI, Prieto-Samsonov DL, Gustavo A, Selman-Housein G (1998). Herbicide-resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. Planta 206: 20-27.

Froger A, Hall JE (2007). Transformation of plasmid DNA into *E. coli* using the heat shock method. J Vis Exp 6: 253.

He X, Miyasaka SC, Fitch MM, Moore PH, Zhu YJ (2008). *Agrobacterium tumefaciens*-mediated transformation of taro (*Colocasia esculenta* (L.) Schott) with a rice chitinase gene for improved tolerance to a fungal pathogen *Sclerotium rolfsii*. Plant Cell Rep 27: 903-909.

Hussnain Z, Afghan S (2006). Impact of Major Cane Diseases on Sugarcane Yield and Sugar Recovery. Annual Report. Jhang, Pakistan: Shakarganj Sugar Research Institute.

Iqbal MM, Nazir F, Ali S, Asif MA, Zafar Y, Iqbal J, Ali GM (2012). Over expression of rice chitinase gene in transgenic peanut (*Arachis hypogaea* L.) improves resistance against leaf spot. Mol Biotechnol 50: 129-136.

Jabeen N, Chaudhary Z, Gulfraz M, Rashid H, Mirza B (2015). Expression of rice chitinase gene in genetically engineered tomato confers enhanced resistance to fusarium wilt and early blight. Plant Pathol J 31; 252.

Jayashree J, Selvi A, Nair N (2010). Characterization of resistance gene analog polymorphisms in sugarcane cultivars with varying levels of red rot resistance. Electronic Journal of Plant Breeding 1: 1191-1199.

Joung YH, Kamo K (2006). Expression of a polyubiquitin promoter isolated from *Gladiolus*. Plant Cell Rep 25: 1081-1088.

Kaur A, Gill M, Gill R, Gosal S (2007). Standardization of different parameters for 'particle gun' mediated genetic transformation of sugarcane (*Saccharum officinarum* L.). Indian J Biotechnol 6: 31-34.

Khan A, Nasir IA, Tabassum B, Aaliya K, Tariq M, Rao AQ (2016). Expression studies of chitinase gene in transgenic potato against *Alternaria solani*. Plant Cell Tiss Org 128: 563-576.

Khan HMWA, Awais M, Raza W, Zia A (2011). Identification of sugarcane lines with resistance to red rot. Pak J Phytopathol 23: 98-102.

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

Malathi P, Viswanathan R (2013). Role of microbial chitinase in the biocontrol of sugarcane red rot caused by *Colletotrichum falcatum* Went. Electronic Journal of Plant Breeding 6: 17-23.

Mohanraj D, Padmanaban P, Karunakaran M (2003). Effect of phytotoxin of *Colletotrichum falcatum* Went. (*Physalospora tucumanensis*) on sugarcane in tissue culture. Acta Phytopathol Entomol Hung 38: 21-28.

Mondal KK, Chatterjee SC, Viswakarma N, Bhattacharya RC, Grover A (2003). Chitinase-mediated inhibitory activity of *Brassica* transgenic on growth of *Alternaria brassicae*. Current Microbiol 47: 171-173.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Mustafa G, Khan MS (2012). Prospecting the utility of antibiotics as lethal selection agents for chloroplast transformation in sugarcane. Int J Agric Biol 14: 307-310.

Nasir IA, Tabassum B, Qamar Z, Javed MA, Tariq M, Farooq AM, Butt SJ, Qayyum A, Husnain T (2014). Herbicide-tolerant sugarcane (*Saccharum officinarum* L.) plants: an unconventional way of weed removal. Turk J Biol 38: 439-449.

Nayyar S, Sharma BK, Kaur A, Kalia A, Sanghera GS, Thind KS, Yadav IS, Sandhu JS (2017). Red rot resistant transgenic sugarcane developed through expression of β-1,3-glucanase gene. PLoS One 12: e0179723.

Rott P (2000). A Guide to Sugarcane Diseases. Versailles, France: Editions Quae.

Sengar A, Thind K, Kumar B, Pallavi M, Gosal S (2009). In vitro selection at cellular level for red rot resistance in sugarcane (*Saccharum* sp.). Plant Growth Regul 58: 201-209.

Sharma R, Tamta S (2015). A review on red rot: the cancer of sugarcane. J Plant Pathol Microbiol S1: 003.

Shin S, Mackintosh CA, Lewis J, Heinen SJ, Radmer L, Dill-Macky R, Muehlbauer GJ (2008). Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. J Exp Bot 59: 2371-2378.

Souza TP, Dias RO, Silva-Filho MC (2017). Defense-related proteins involved in sugarcane responses to biotic stress. Genet Mol Biol 40: 360-372.

Su Y, Xu L, Fu Z, Yang Y, Guo J, Wang S, Que Y (2014). ScChi, encoding an acidic class III chitinase of sugarcane, confers positive responses to biotic and abiotic stresses in sugarcane. Int J Mol Sci 15: 2738-2760.

Su Y, Xu L, Wang S, Wang Z, Yang Y, Chen Y, Que Y (2015). Identification, phylogeny, and transcript of chitinase family genes in sugarcane. Sci Rep 5: 10708.

Tang S, Heesacker A, Kishore VK, Fernandez A, Sadik ES, Cole G, Knapp SJ (2003). Genetic mapping of the gene for resistance to race E in sunflower. Crop Sci 43: 1021-1028.

Viswanathan R (2012). Molecular basis of red rot resistance in sugarcane. Functional Plant Science and Biotechnology 6: 40-50.

Viswanathan R (2017). Pathogen virulence in sugarcane red rot pathogen versus varieties in cultivation: classical case of loss in virulence in the pathotype CF06 (Cf671). Sugar Tech 19: 293-299.

Viswanathan R, Samiyappan R (2002). Induced systemic resistance by fluorescent pseudomonads against red rot disease of sugarcane caused by *Colletotrichum falcatum*. Crop Prot 21: 1-10.

Wang M, Li Z, Matthews PR, Upadhyaya NM, Waterhouse PM (1997). Improved vectors for Agrobacterium tumefaciens-mediated transformation of monocot plants. In: International Symposium on Biotechnology of Tropical and Subtropical Species, Part 2.

Yevtushenko DP, Romero R, Forward BS, Hancock RE, Kay WW, Misra S (2005). Pathogen-induced expression of a cecropin A-melittin antimicrobial peptide gene confers antifungal resistance in transgenic tobacco. J Exp Bot 56: 1685-1695.