Development of transgenic finger millet (*Eleusine coracana* (L.) Gaertn.) resistant to leaf blast disease

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Finger millet plants conferring resistance to leaf blast disease have been developed by inserting a rice chitinase (*chi*11) gene through *Agrobacterium*-mediated transformation. Plasmid pHyg-Chi.11 harbouring the rice chitinase gene under the control of maize ubiquitin promoter was introduced into finger millet using *Agrobacterium* strain LBA4404 (pSB1). Transformed plants were selected and regenerated on hygromycin-supplemented medium. Transient expression of transgene was confirmed by GUS histochemical staining. The incorporation of rice chitinase gene in R_0 and R_1 progenies was confirmed by PCR and Southern blot analyses. Expression of chitinase gene in finger millet was confirmed by Western blot analysis with a barley chitinase antibody. A leaf blast assay was also performed by challenging the transgenic plants with spores of *Pyricularia grisea*. The frequency of transient expression was 16.3% to 19.3%. Stable frequency was 3.5% to 3.9%. Southern blot analysis confirmed the integration of 3.1 kb chitinase gene. Western blot analysis detected the presence of 35 kDa chitinase enzyme. Chitinase activity ranged from 19.4 to 24.8. In segregation analysis, the transgenic R_1 lines produced three resistant and one sensitive for hygromycin, confirming the normal Mendelian pattern of transgene segregation. Transgenic plants showed high level of resistance to leaf blast disease compared to control plants. This is the first study reporting the introduction of rice chitinase gene into finger millet for leaf blast resistance.

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1. Introduction

Millets are staple foods that supply a major portion of calories and protein to large segments of populations in the semi-arid tropical regions of Africa and Asia (O'Kennedy *et al.* 2006). Finger millet (*Eleusine coracana* (L.) Gaertn.) is the primary food source for millions of people in tropical dry land regions. It also has nutritional qualities superior to that of rice and is on par with wheat (Latha *et al.* 2005). The crop is also adapted to a wide range of tropical soils, ranging from red lateritic to sandy loams and black heavy vertisols (Dida and Dvos 2006). It is estimated that some 10% of the world's 30 million tons of millet produced is finger millet (Dida *et al.* 2008). Finger millet is used in various food preparations. It is usually converted into flour and made into cakes, bread and other bakery products. The sprouted seeds are also nutritious and easily digested. The grain may also be malted and a flour of the malted grain is used as a nourishing food for infants (Mgonja *et al.* 2007).

Finger millet is vulnerable to leaf blast caused by *Pyricularia grisea* (Harinarayana 1986). High seed yield loss (>50%) is reported (Sastri 1989). This disease occurs almost every year during rainy season but the extent of crop loss depends on the severity and the time of onset. The chemicals recommended for controlling the spread of blast disease are Mancozeb, Carbendazin and Kitazin (Viswanath and Seetharam 1986). These synthetic fungicides pose a great threat to biodiversity by disturbing wildlife, such as birds, honey bees, fish and aquatic invertebrates. They also cause adverse effects on soil microorganisms and disrupt nitrogen cycle (Kinney *et al.* 2005). Development of fungal-resistant finger millet by transferring genes such as chitinase is one of the best options available today to overcome fungal attack and improve the yield of finger millet. So there

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is an urgent need for genetic improvement of finger millet by transferring fungal resistance genes (Ceasar and Ignacimuthu 2009).

Only a limited number of studies have been conducted on transformation of finger millet. Preliminary work on finger millet transformation was performed by Gupta *et al.* (2001), who compared the efficiency of five gene promoters for the expression of the GUS reporter gene. A transgenic finger millet resistant to leaf blast disease was developed by Latha *et al.* (2005). Antifungal protein (PIN) from prawn was chemically synthesized and cloned into plasmid pPin35S and the bar reporter gene was cloned into plasmid pBar35S; both the genes were under the control of CaMV35S promoter. The transformed plants were selected on phosphinothricin-supplemented medium. Stable integration and expression of the PIN gene were confirmed by Southern and Northern blot analyses (Latha *et al.* 2005). This is the only study on transformation of finger millet with fungal resistance gene. Several attempts were made on other cereals for the development of fungal-resistant transgenic plants. Rice chitinase gene has been most frequently used for the production of fungal-resistant transgenic plants. Crop plants engineered with chitinase gene have been found to be effective in controlling the fungal diseases (rice – Datta *et al.* 2001; Kumar *et al.* 2003; Kalpana *et al.* 2006; Nandakumar *et al.* 2007; Sridevi *et al.* 2008; cucumber – Tabei *et al.* 1998; strawberry – Asao *et al.* 1997; American ginseng – Chen and Punja 2002; mustard – Grison *et al.* 1996; peanut – Rohini and Rao 2001). No report is available on transformation of finger millet with chitinase gene. So, the present study was aimed at introducing rice chitinase gene (*chi*11) into finger millet using *Agrobacterium*-mediated transformation.

2. Materials and methods

2.1 *Plant material and tissue culture*

The finger millet genotype GPU 45, obtained from the University of Agricultural Sciences, Bangalore, was used for the introduction of rice chitinase gene. A tissue culture protocol (Ceasar and Ignacimuthu 2008) was used for the preparation of shoot apex explant, somatic embryogenesis and regeneration. Callus induction and somatic embryogenesis were obtained on Murashige and Skoog (MS) (1962) medium containing 3% sucrose, 18.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.3 µM kinetin (Kn) and 0.8% agar. Matured somatic embryos were regenerated on MS medium supplemented with 3% sucrose, 4.5 µM thidiazuran (TDZ), 4.6 µM Kn and 0.8% agar. The regenerated plants were hardened and transferred to the field after acclimatization.

2.2 *Plasmid construction*

The rice chitinase gene (Huang *et al.* 1991) under maize ubiquitin promoter was obtained as a 3.1 kb *Hin*dIII fragment from plasmid pCAMBAR.Chi.11 (kindly provided by Dr S Muthukrishnan, Department of Biochemistry, Kansas State University). This fragment was subcloned into a binary plasmid pCAMBIA1301 (CAMBIA, Canberra, Australia) and the resulting plasmid was designated as pHyg-Chi.11 (figure 1). This plasmid had hygromycin phosphotransferase (*hph*) gene as the plant selection marker and β-glucuronidase gene with intron (*int-gus*) as the reporter. This binary vector was mobilized into *A. tumefaciens* strain LBA4404 (pSB1) (Ishida *et al.* 1996) by triparental mating (Ditta *et al.* 1980).

2.3 *Bacterial culture and transformation conditions*

An *Agrobacterium*-mediated transformation protocol developed in our lab (Ceasar and Ignacimuthu 2011) was used in this study. Overnight grown suspension culture of *Agrobacterium* strain LBA4404 (pHyg-Chi.11) was prepared by inoculating 30 mL of liquid YEP (Sambrook *et al.* 1989) medium supplemented with 50 mg/L kanamycin and 10 mg/L rifampicin in a 125 mL flask with a single colony. The culture was maintained at 26°C on an orbital shaker (185 rpm) in the dark. After 12 h, an optical density (OD_{600}) of 0.6–0.8 was obtained (Spectrophotometer, Hitachi, Japan); it was centrifuged at 10,000 rpm at 4°C for

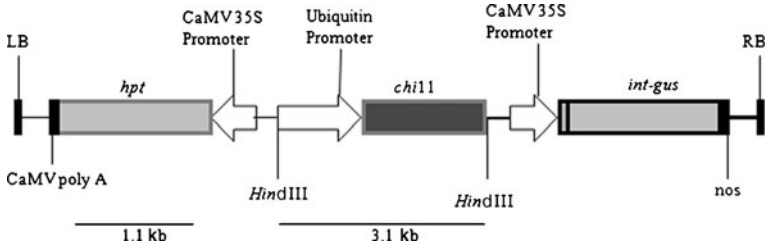


Figure 1. T-DNA region of the plasmid pHyg-Chi.11. This plasmid has hygromycin phosphotransferase (*hph*) gene as a plant selection marker and β-glucuronidase gene with intron (*int-gus*) as a reporter. The recognition sites of *Hin*dIII and probe regions for Southern hybridization of *chi*11and *hph* genes are indicated.

10 min and the pellet was re-suspended in liquid somatic embryogenesis medium supplemented with 100 µM acetosyringone. The bacterial suspension (50 mL) with an optical density (OD_{600}) of 0.8 was obtained. Fifty shoot apex explants were inoculated for 30 min in this suspension. The explants were then co-cultivated on somatic embryogenesis medium supplemented with 100 µM acetosyringone for 3 days with air-drying. The air-drying was performed by placing the explants on the filter paper that was kept over the co-cultivation medium (Ceasar and Ignacimuthu 2011).

2.4 *Selection and regeneration of transformed finger millet*

After co-cultivation for 3 days in dark, the explants were transferred to selection (somatic embryogenesis) medium supplemented with 250 mg/L cefotaxime and 25 mg/L hygromycin. Explants were closely observed for the production of hygromycin-resistant tissues or necrosis; the explants producing resistant tissues were separated and subcultured in the same medium for 7 weeks with changes into the fresh medium every 2 weeks. After 7 weeks somatic embryos were produced and these were transferred to the regeneration medium containing 250 mg/L cefotaxime and 25 mg/L hygromycin. Hygromycin-resistant plants were recovered from the selection medium, hardened and established in the field.

2.5 *GUS histochemical assay*

Explants that grew on selection medium were used for GUS histochemical assay (Jefferson *et al.* 1987) to check for transient expression of GUS gene. Hygromycin-resistant explants were incubated with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) substrate solution for the analysis of GUS expression. The explants were incubated in sodium phosphate buffer (50 mM $NaPO_4$ pH 6.8) that contained 1% Triton X-100 at 37°C for 1 h. The reaction mixture was incubated overnight at 37°C for blue colour development.

2.6 *PCR analysis*

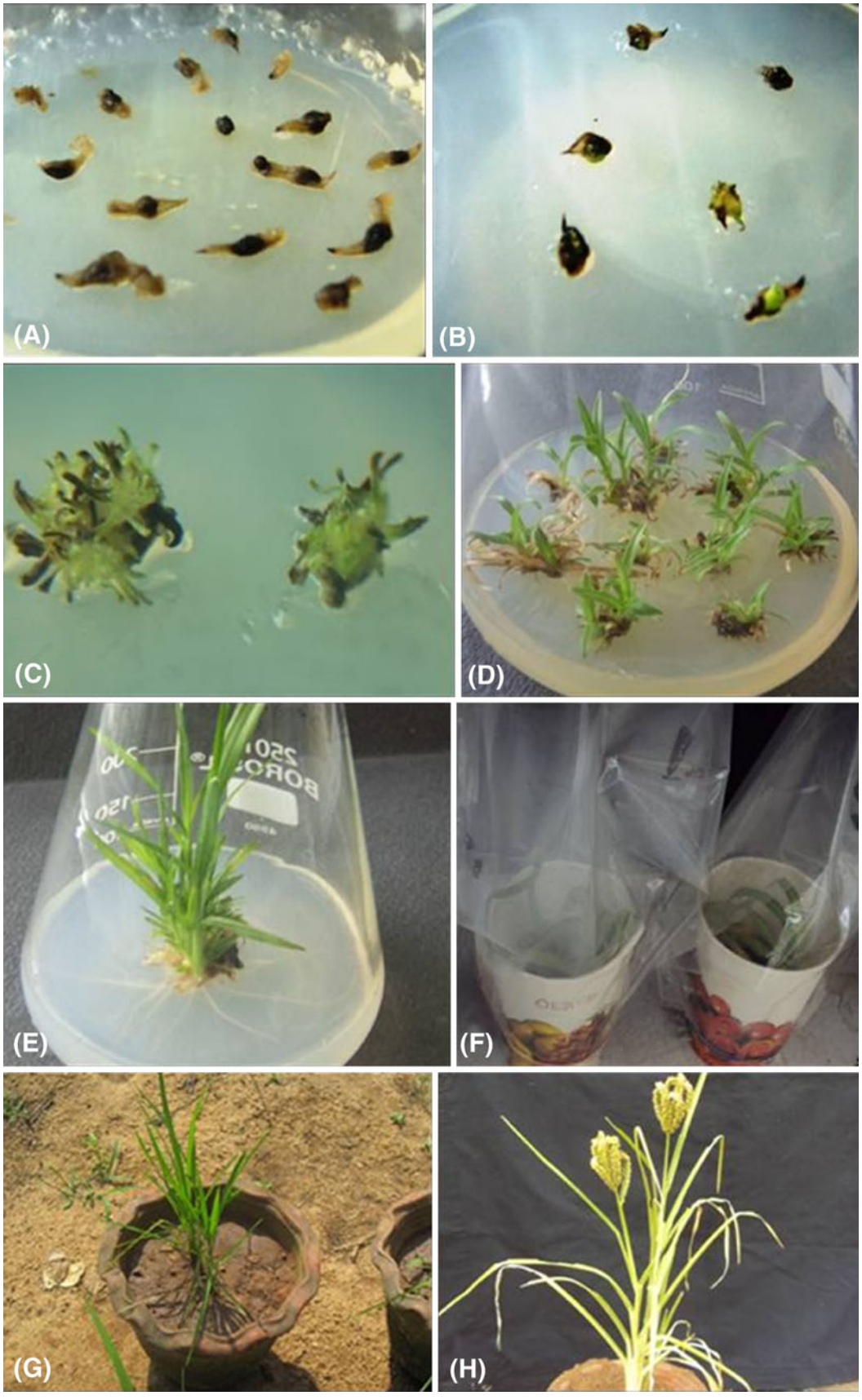
Genomic DNA was isolated from leaf tissues of hygromycin resistant lines from R_0 and R_1 progenies using the Doyle and Doyle (1990) method. DNA was amplified with *chi*11 gene specific primers (forward: 5′ GCTTCTACACCTACGACGCCTT 3′; reverse: 5′ GTAGCGCTTGTAGAACCCGATC 3′) to check the integration of *chi*11 gene. PCR was carried out in a volume of 25 µL reactions consisting of 1 µL template DNA (40–80 ng), 2.5 µL 10X PCR buffer, 2.5 µL $MgCl_2$ (2.5 mM), 0.5 µL of dNTPs (10 mM), 2 µL (7.5 pmol) each primer, 1 µL *Taq* polymerase (0.5 U) (BangaloreGenei Pvt Limited, India) and 13.5 µL sterile nuclease-free water. The amplification was carried out in a DNA thermal cycler (Eppendorf, Germany) with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 2 min and extension at 72°C for 3 min; final extension was carried out at 94°C for 7 min. The amplified samples were electrophoresed in 1% agarose gel and analysed.

2.7 *Southern blotting*

Southern analysis was performed as described by Southern (1975). Labelling of probes, hybridization and detection steps were performed as per the instructions of the manufacturer (Fermentas Life Sciences, USA). Genomic DNA of hygromycin-resistant finger millet was digested with the restriction enzyme *Hin*dIII which released 3.1 kb fragment of *chi*11 along with ubiquitin promoter from the middle portion of T-DNA. This digestion also released *hph* gene along with plant DNA at the left border region of T-DNA (figure 1). The digested DNA was separated on a 0.8% agarose gel and transferred onto nitrocellulose membrane (BangaloreGenei, India) using capillary transfer with 0.4 M NaOH. The coding regions of *chi*11 and *hph* genes were used for the preparation of probe (figure 1) using biotin-11-dUTP deca label DNA labelling kit (Biotin Deca Label DNA Labeling Kit, Fermentas Life Sciences, USA). The blot was subjected to detection by overnight colour development using biotin chromogenic detection kit (Biotin Chromogenic Detection Kit, Fermentas Life sciences, USA) following the manufacturer's instructions.

2.8 *Western blot analysis*

Western blot analysis was performed to detect the expression of chitinase enzyme in R_0 and R_1 progenies. Total protein was isolated from young leaves of 40-day-old R_0 progeny and leaf (15-day-old) and stem (45-day-old) tissues of R_1 progeny. One gram of leaf material was homogenized in 6 mL of 1 M sodium phosphate buffer (pH 7.0), centrifuged at 12000*g* for 20 min at 4°C and the supernatant was collected. This was used as crude enzyme source for Western analysis and for the assay of chitinase activity. The total protein was quantified by Bradford (1988) method. Twenty microgram of protein samples were separated in 12% SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane in Tris-Glycine electroblotting transfer buffer using an electro blotting apparatus (Bangalore Genei Pvt Ltd, Bangalore, India). The membrane was blocked using bovine serum albumin protein and Tween-20-buffered saline and probed with


(A)
(B)
(C)
(D)
(E)
(F)
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chitinase antibody, (a polyclonal rabbit antibody raised against barley chitinase, kindly provided by Dr S Muthukrishanan, Kansas State University) at a dilution of 1:1000 v/v and goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Bangalore Genei) at a dilution of 1:2000. Detection was carried out using Tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) solution (Helini Biomolecules, Chennai, India).

2.9 *Chitinase enzyme assay*

Chitinase enzyme assay was performed to check the level of chitinase produced in hygromycin-resistant plants and compared with control plants. The colorimetric assay of chitinase was carried out following the method of Mauch *et al.* (1988) using colloidal chitin as the substrate. The reaction mixture consisted of 10 µL 0.1 M sodium acetate buffer (pH 4.0), 0.4 mL plant enzyme extract and 0.1 mL colloidal chitin (10 mg). After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 1000*g* for 3 min. An aliquot of the supernatant (0.3 mL) was pipetted into a glass reagent tube containing 30 µL 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µL 3% (w/v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µL 0.1 M sodium borate buffer (pH 9.8). The mixture was held in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. After the addition of 2 mL *p*-dimethylaminobenzaldehyde (DMAB), the mixture was incubated for 20 min at 37°C and the absorbance was read at 585 nm. *N*-acetylglucosamine (GlcNAc) was used as a standard. The enzyme activity was expressed as nmol GlcNAc equivalents/min/g fresh weight.

2.10 *Leaf blast assay*

Pyricularia grisea culture was procured from the Department of Plant Pathology, Tamil Nadu Agriculture University, Coimbatore, India. These cultures were subcultured on potato dextrose agar liquid medium and incubated at 28°C. Fungal spores were prepared following the method of Radjacommare *et al.* (2004). The maize stem bits were infected with *P. grisea* cultures by introducing 5–10 fungal discs of *P. grisea* and incubated at 28°C. After 10 days, 50 mL of distilled water was added to the infected maize stem bits and filtered. The filtrates containing fungal spores were then transferred into a sprayer and 200 mL of spores were sprayed on 40- to 45-day-old R_1 plants for infection. These plants were then tightly covered by polythene sheet for 2–3 days and the outer surface was sprinkled with water to maintain high humidity of 95±2°C for quick infection of the pathogen. The symptoms developed were observed after 7 days. The spots developed were monitored closely, measured and compared with that of control plants. The resistance/susceptibility of transgenic and untransformed control plants were scored following the method of Latha *et al.* (2005). The plants were graded 0–9 on the basis of the level of resistance; plants falling in the range of 0 to 4 were graded as resistant, while plants falling in the range of 5 to 9 were scored as susceptible.

2.11 *Segregation analysis of transgene in R_1 progenies*

A segregation analysis was performed to check the pattern of inheritance. Seeds collected from selfed R_0 plants were screened for hygromycin resistance; the seeds were germinated initially on MS basal medium containing 3% sucrose (solidified using 0.8% agar) and placed in dark. The germinated seedlings were then transferred to the same medium supplemented with 25 mg/L hygromycin and placed in light. After 10 days, seedlings were scored for hygromycin resistance (Hyg^S and Hyg^R) and the data were validated using χ^2 test. From this, segregation was analyzed.

2.12 *Data analysis*

All experiments were carried out in a completely randomized design. Each experiment was replicated three times, each replicate consisting of more than 10 explants based on the experiment. The mean frequency (%) of transient GUS expression (number of explants producing blue spots/total number of explants×100) was calculated 3 weeks after infection and mean frequency (%) of stable transformation (number of transgenic plants regenerated/total number of explants×100) was calculated after 7 weeks under regeneration on hygromycin. Data were analysed statistically (ANOVA or χ^2 test) and the mean and standard deviation were calculated for each experiment. The least significance difference (LSD) was calculated to find the

◀ **Figure 2.** Selection, regeneration and field transfer of finger millet genotype GPU 45 transformed with rice chitinase gene (*chi*11) using *A. tumefaciens* LBA4404 (pHyg.Chi.11). (**A**) negative control (non-transformed explants); (**B**) production of greenish structures from somatic embryos on selection (somatic embryogenesis) medium; (**C**) shoot induction from transformed somatic embryos on selection (regeneration) medium; (**D**) shoot elongation after subculture in selection (regeneration) medium; (**E**) root induction and whole plant formation in selection (regeneration) medium; (**F**) hardening of plants recovered from selection (regeneration) medium; (**G**) hardened plant and (**H**) field establishment and seed setting.

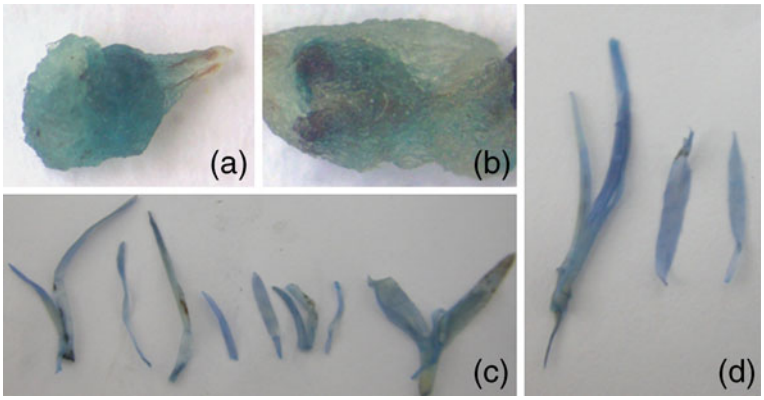

Figure 3. GUS expression in explants of finger millet genotype GPU 45 transformed with rice chitinase gene (*chi*11) using *A. tumefaciens* LBA4404 (pHyg.Chi.11). (**a** and **b**) GUS expression in shoot apex derived callus, obtained 15 days after infection with *Agrobacterium*; (**c**) GUS expression in leaf, shoot and stem regions of R_0 progeny plants recovered from the selection medium containing hygromycin and (**d**) GUS expression in various regions of R_1 progeny.

significance. The SPSS Inc. software was used for data analyses.

3. Results

Leaf-blast-resistant finger millet plants were developed by inserting rice chitinase gene through *Agrobacterium*-mediated transformation. The plants were regenerated on hygromycin supplemented medium; GUS assay, PCR, Southern blot, Western blot and chitinase assay were performed to confirm the integration of chitinase gene. Leaf blast assay was also performed to assess the disease resistance.

3.1 *Selection and regeneration of transformed plants*

The explants were selected on hygromycin (25 mg/L) after 3 days of co-cultivation with *A. tumefaciens* LBA4404 (pHyg-Chi.11). The transformed explants produced hygromycin-resistant calli, somatic embryos and later shoots in the regeneration medium (figure 2a–f). The hygromycin-resistant plants were recovered from selection medium, hardened and transferred to the field (figure 2g–h). The survival frequency was more than 95%. The regenerants grew well and did not show any phenotypic variation compared to normal plants. The growth features and seed setting were also normal.

3.2 *GUS histochemical assay*

All explants that survived on the selection medium produced blue colour after overnight incubation with X-gluc substrate solution. Intense blue colour was seen in different tissues after co-cultivation with *A. tumefaciens* LBA4404 (pHyg.Chi.11). The R_1 progeny plants also produced intense blue colour, confirming the transient expression of GUS gene (figure 3). The frequency of transient expression (16.3% to 19.3%) and stable transformation (3.5 to 3.9%) varied among four different transformation experiments (table 1).

3.3 *PCR analysis of hygromycin-resistant plants*

PCR was performed to confirm the presence of chitinase gene. The amplified band of 0.584 kb was obtained in all

Table 1. Transformation of finger millet genotype GPU 45 with rice chitinase gene (*chi*11) using LBA4404 (pHyg.Chi.11)

Experiment number	Number of explants	Number of GUS positive explants	Frequency of transient GUS expression (%)	Frequency of stable transformation (%)
1	105	61	19.3±0.9	3.8±0.6
2	104	55	17.6±1.1	3.5±0.4
3	102	57	18.6±0.9	3.9±0.3
4	110	54	16.3±0.5	3.6±0.5

Values are mean ± SD of three replications.

the four hygromycin-resistant lines of R_0 progeny plants (figure 4a) confirming the successful introduction of rice chitinase gene. PCR performed with genomic DNA of R_1 progeny plants also produced positive response for *chi*11 gene amplification (figure 4b). This confirmed the successful integration of rice chitinase (*chi*11) gene.

3.4 *Southern blot analysis*

To further confirm the integration of chitinase gene, PCR positive plants were analysed by Southern hybridization. Upon hybridization and detection, all the four hygromycin-resistant lines tested showed the expected band size of 3.1 kb (figure 5a) very clearly and distinctly; the probe detected rice chitinase gene (*chi*11) alone. The inheritance of chitinase gene in R_1 progeny plants was also checked by

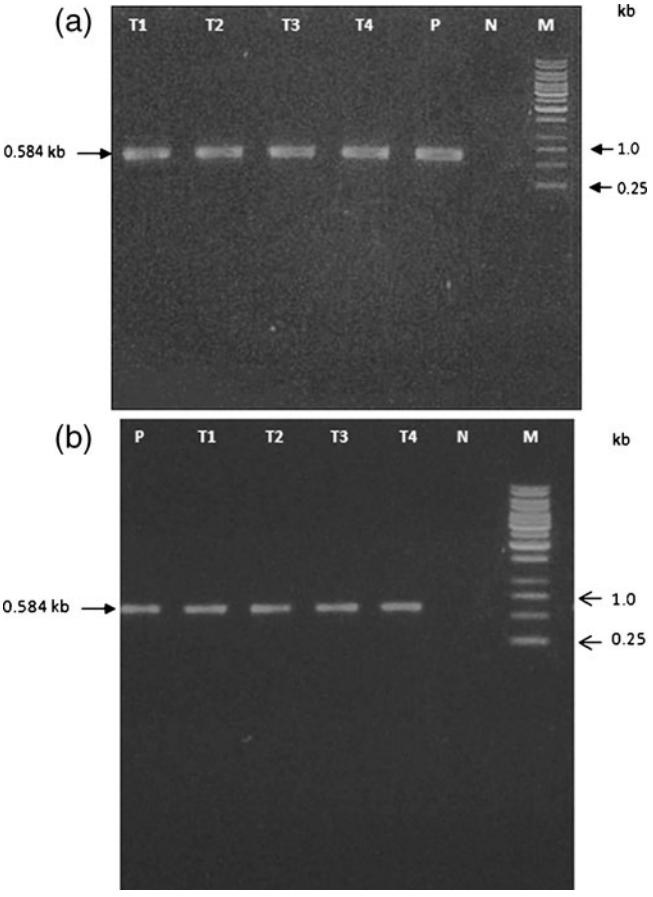

Figure 4. Amplification of *chi*11 gene from genomic DNA, isolated from R_0 (**a**) and R_1 (**b**) progenies of finger millet plants of genotype GPU 45 resulting from infection with *A. tumefaciens* LBA4404 (pHyg.Chi.11). Lane M, 1 kb marker; lane N, non-transformed plant DNA; lanes T1–T4, transformed plants (plant numbers T1, GPU 45-1; T2, GPU45-12; T3, GPU 45-19, T4, GPU 45-25); lane P, positive control (pHyg.Chi.11plasmid).

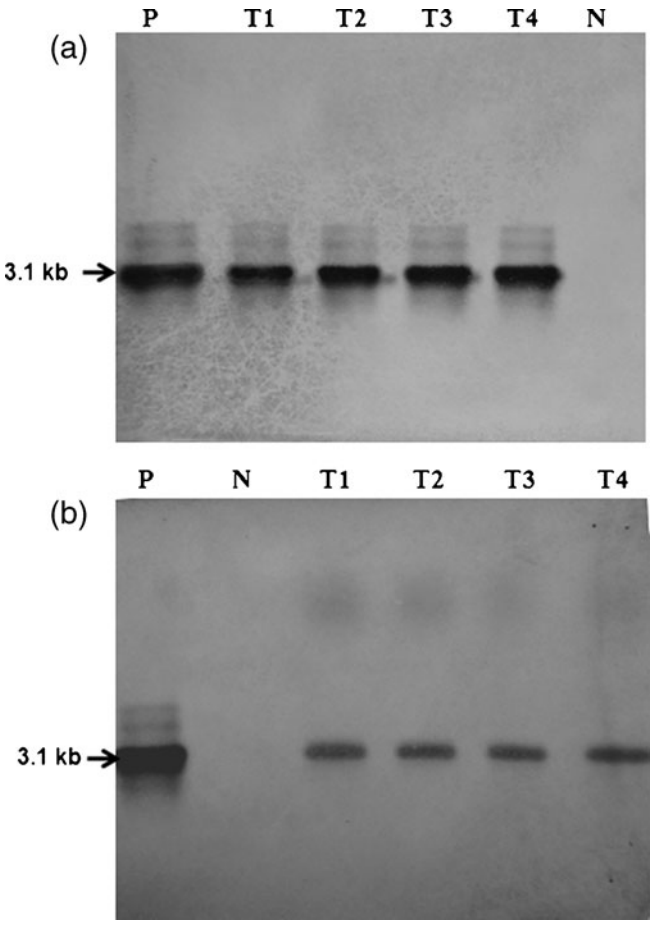

Figure 5. Southern blot analysis of four R_0 (**a**) and R_1 (**b**) progenies of finger millet plants of genotype GPU 45 resulting from infection with *A. tumefaciens* LBA4404 (pHyg.Chi.11). Genomic DNA was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nitrocellulose membrane, and allowed to hybridize to the *chi*11 gene probe. Lane P, plasmid (pHyg.Chi.11) DNA digested with *Hin*dIII; lanes T1–T4, transformed finger millet plants (plant numbers T1, GPU 45-1; T2, GPU 15-12; T3, GPU 45-19, T4, GPU 45-25); lane N, non-transformed plant DNA.

Southern analysis. Four R_1 progenies were analysed and all these four lines produced 3.1 kb band (figure 5b). The pattern of transgene integration in these four lines was calculated using *hph* probe in a separate Southern analysis; two lines had a single copy and other two lines had two copies of transgene integration in R_0 and R_1 progenies (figure 6a and b).

3.5 *Western blot analysis*

The presence of chitinase protein in PCR and Southern positive plants was analysed by Western blot using a barley chitinase

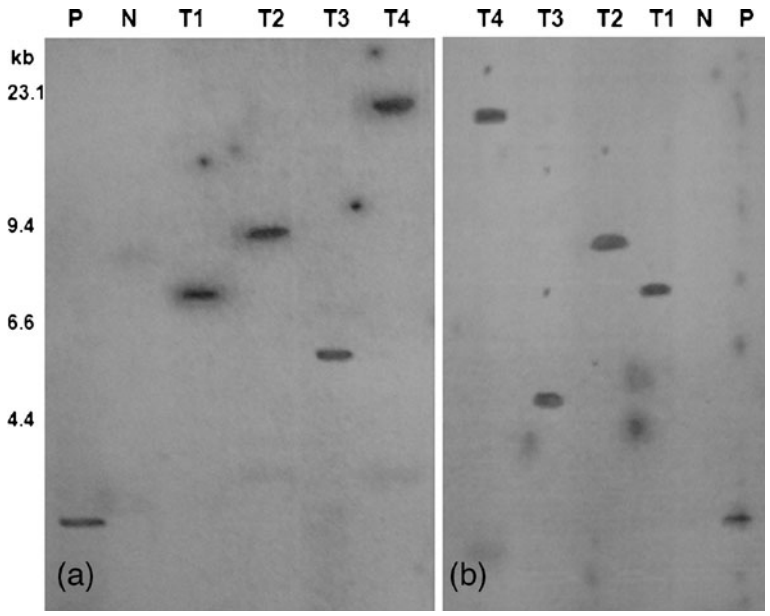

Figure 6. Southern blot analysis of four R_0 (**a**) and R_1 (**b**) progenies of finger millet plants of genotype GPU 45 resulting from infection with *A. tumefaciens* LBA4404 (pHyg.Chi.11).Genomic DNA was digested with *Hind*III, fractionated by electrophoresis, transferred to a Nitrocellulose membrane, and allowed to hybridize to the *hph* gene probe. Lane N, non-transformed plant DNA; lanes T1–T4, transformed finger millet plants (plant numbers T1, GPU 45-1; T2, GPU 15-12; T3, GPU 45-19, T4, GPU 45-25).

polyclonal antibody. The polyclonal antibody detected 35 kDa chitinase in all the four hygromycin resistant lines of R_0 progeny plants tested (figure 7a). In addition to 35 kDa signal, another band was detected at 27–28 kDa in the same blot. The leaf and stem tissues obtained at different stages of R_1 progeny also produced same signals at 35 kDa and 28 kDa, confirming the inheritance and expression of chitinase gene (figure 7b). This analysis confirmed the constitutive expression of rice chitinase gene in hygromycin-resistant lines.

3.6 *Assay of chitinase enzyme activity*

The chitinase activity was significantly higher in hygromycin-resistant plants compared to non-transformed control plants (table 2). The enzyme activities in hygromycin-resistant lines ranged from 19.4 to 24.8 (table 2); maximum enzyme activity (24.8 units) was observed in hygromycin-resistant line 24.

3.7 *Leaf blast assay*

Disease symptoms were quickly developed on control plants after spraying with fungal spores. Many small spots of fungal infection were observed on leaves of control plants (figures 8 and 9). The hygromycin-resistant plants with inserted chitinase gene (*chi*11) did not get infected quickly by *P. grisea* and exhibited noticeable resistance to leaf blast disease (table 3). Hygromycin-resistant plants produced only fewer lesions (0–6.7) compared with the control (15–16). The lesions formed on the resistant leaves were also localized at the point of infection, whereas lesions developed on the susceptible control leaves expanded quickly in size, leading to death of seedlings within 25–30 days after the spraying of the fungal spores. High level of resistance was conferred by transgenic finger millet plants constitutively expressing rice chitinase gene after transformation by *A. tumefaciens*.

3.8 *Segregation analysis of the transgene in finger millet*

A segregation analysis was performed to check the pattern of transgene segregation in R_1 progenies of transformed plants. Four different hygromycin-resistant lines of GPU 45 were screened on hygromycin (25 mg/L)-supplemented medium to test the resistance and susceptibility of R_1 seeds. The chi-square test was used to validate the data (table 4). All the four hygromycin-resistant lines of GPU 45 gave R_1 segregation of Hyg^R/Hyg^S in the ratio of 3:1, confirming the normal Mendelian pattern of transgene segregation in R_1 progenies.

4. Discussion

Rice chitinase gene has been introduced into finger millet by *Agrobacterium*-mediated transformation to confer resistance to leaf blast. A major objective of plant biotechnology is to improve the yield of crop plants by conferring resistance

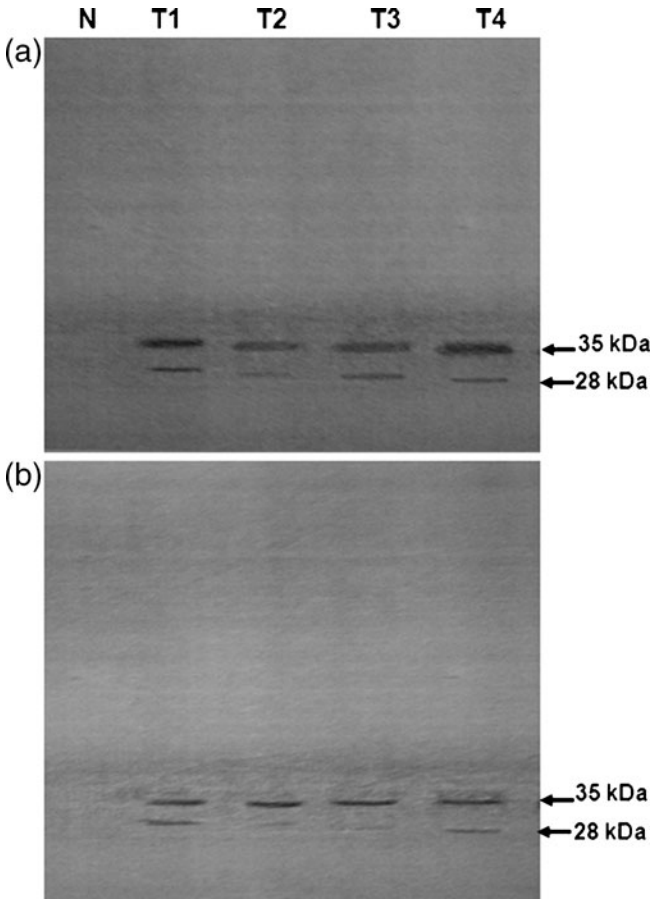

Figure 7. Western blotting analysis for chitinase expression in finger millet plants of genotype GPU 45 transformed with *A. tumefaciens* LBA4404 (pHyg.Chi.11). (**a**) R_0 progeny. Lane N, Non-transformed plant. Lanes T1–T4, transformed plants (plant numbers T1, GPU 45-1; T2, GPU 15-12; T3, GPU 45-19, T4, GPU 45-25). (**b**) R_1 progeny Lane N, Non-transformed plant. Lanes T1–T2, protein isolated from leaf tissues of 15-day-old plant (plant numbers T1, GPU 45-1; T2, GPU 15-25). Lanes T3–T4, protein isolated from stem portion of 45-day-old plant (plant numbers T3, GPU 45-1; T2, GPU 15-25).

against biotic stresses like insect damage and fungal and viral diseases through alien gene transfer. Fungal disease is a major constraint in the crop production due to high yield loss. Significant yield losses occur in most of the agricultural and horticultural species due to fungal attacks. In Indian context, fungal diseases are rated either the most important or second most important factor contributing to yield losses in major cereal, pulse and oilseed crops (Grover and Gowthaman 2003). Several successful attempts have been made to develop fungal-resistant cereal crops through genetic engineering. The chitinase gene has been considered best candidate for defending crop plants from fungal diseases. So, this gene was inserted into finger millet, which conferred high level of resistance against leaf blast disease.

Table 2. Chitinase activity [nmol (GlcNAc equivalents) g^{-1} min^{-1}] in R_0 progeny of finger millet plants transformed with rice chitinase gene

Plant samples (GPU 45)	Chitinase activity
Non-transformed control	5.8±0.8a
Transgenic line-1	22.7±0.7b
Transgenic line-10	19.4±1.1b
Transgenic line-12	17.6±0.8b
Transgenic line-17	23.7±1.2b
Transgenic line-19	20.6±1.3b
Transgenic line-24	24.8±0.9b
Transgenic line-25	22.2±1.3b
Transgenic line-29	23.7±0.9b

Values are mean±SD of three replications. Means followed by a common letter are not significantly different by LSD test (P=0.05 %).

The rice chitinase gene under ubiquitin promoter was cloned into a binary vector pCAMBIA1301 to obtain a plant expression plasmid pHyg.Chi.11. A similar strategy was followed for cloning and expression of the same gene in rice by Sridevi *et al.* (2006); they used pCAMBIA3301 instead of pCAMBIA1301, which had *pat* (phosphinothricin resistance) gene as the plant selection marker. Rice chitinase gene was put under maize ubiquitin promoter. Ubiquitin promoter has been shown to be highly active in monocots (Christensen and Quail 1996; Rooke *et al.* 2000). This promoter helped for the high level of expression of chitinase in finger millet. In previous studies, plasmids containing rice chitinase under ubiquitin have been used for high level expression of rice chitinase gene in rice (Lin *et al.* 1995; Nishizawa *et al.* 1999; Datta *et al.* 2000, 2001; Baisakh *et al.* 2001; Sridevi *et al.* 2003, 2006, 2008; Kumar *et al.* 2003; Kalpana *et al.* 2006; Nandakumar *et al.* 2007; Sripriya *et al.* 2008). Successful integration of chitinase gene into finger millet was confirmed by performing a Southern blot with genomic DNA (*Hin*dIII digest) of PCR-positive plants. All PCR-positive plants produced signals at 3.1 kb, which was the expected band size of chitinase expression cassette. Southern hybridization analysis offers many advantages in the analysis of transgenic plants (Shitara *et al.* 2004) and is equally informative as real-time PCR analysis (Sridevi *et al.* 2006).

Expression of rice chitinase gene in hygromycin-resistant plants was confirmed by Western blot analysis using barley chitinase anti-rabbit antibody as primary antibody. After detection, a 35 kDa signal was produced in hygromycin-resistant plants in addition to a 28 kDa signal. The additional signal at 28 kDa may be due to proteolytic

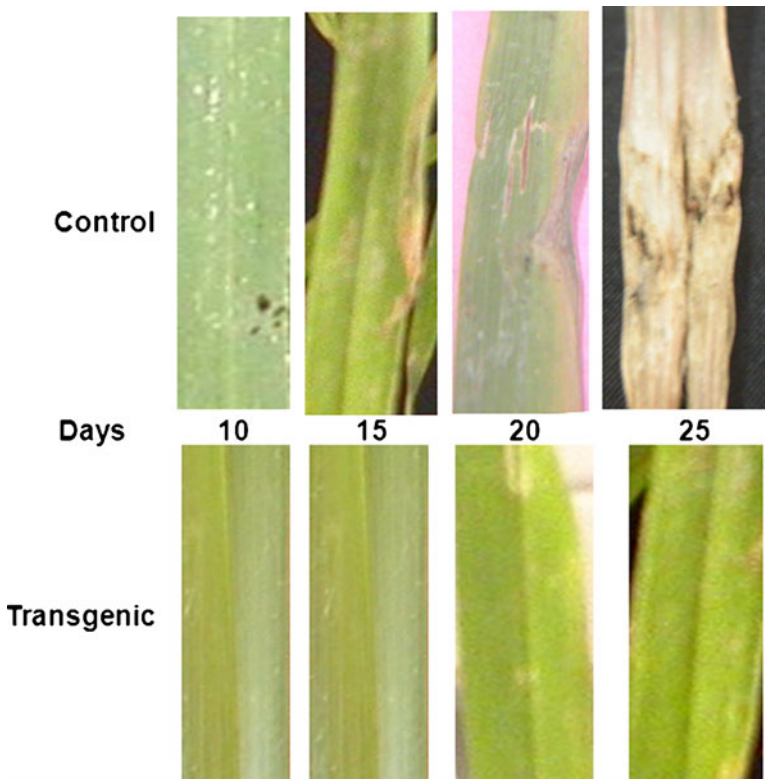

Figure 8. Assessment of leaf blast resistance in R_1 transgenic plants of GPU 45 (GPU45-19) after challenging with *Pyriculariae grisea* spores. The symptoms were noted after respective days of spraying fungal spores.

degradation of chitinase protein (Nandakumar *et al.* 2007). This is in agreement with earlier studies that confirmed the expression of rice chitinase gene in rice by similar strategy and the same bands (35 kDa and 28 kDa) were also observed (Kumar *et al.* 2003; Kalpana *et al.* 2006; Nandakumar *et al.* 2007; Sridevi *et al.* 2008). Barley chitinase antibody specifically recognized rice chitinase protein alone in Western blot analysis. This study further supports the analysis of rice chitinase detection using a barley chitinase antibody as no signal was seen in non-transformed control plants.

Hygromycin-resistant plants showed increased chitinase activity. Chitinase enzyme assay has been routinely performed in analysis of transgenic plants transformed with chitinase gene. The rice plants transformed with chitinase gene exhibited increased activity from 5.8 to 25 units (Nandakumar *et al.* 2007), which was close to the values obtained in this study. In other reports, chitinase activity was reported to increase in transgenic rice plants by 14-fold (Lin *et al.* 1995) and 10- to 62-fold (Sridevi *et al.* 2008). A similar response of increased chitinase activity was observed in other transgenic plants expressing chitinase gene (Kumar *et al.* 2003; Kalpana *et al.* 2006; Nandakumar *et al.* 2007; Sripriya *et al.* 2008).

Leaf blast assay showed that the transformed plants conferred high level of resistance to leaf blast disease. In a previous attempt, fungal-resistant transgenic finger millet plants were developed by inserting an antifungal protein (*PIN*) gene of prawn through biolistic method of transformation (Latha *et al.* 2005). This is the only available previous study for genetic engineering of finger millet for fungal resistance. We followed the same method (Latha *et al.* 2005) for leaf blast assay. It was found that the level of leaf blast resistance was higher in transgenic finger millet plants expressing rice chitinase gene compared with those expressing *PIN* gene. Transgenic plants expressing *PIN* gene exhibited resistance scale of 0–4. In the present study, chitinase expressing plants exhibited resistance scale of 0–3. The size of the lesions were 0.1–0.5 mm in *PIN*-expressing plants, while in chitinase-expressing plants the lesions were only of 0.1–0.4 mm. This might be due to immediate degradation of fungal cell wall material chitin by overexpression of chitinase leading to the control of lesion formation and its spread. Chitinase can also inhibit fungal growth by causing lysis of hyphal tips (Mauch *et al.* 1988).

Radjacommare *et al.* (2004) described the purification of a 57 kDa chitinase from finger millet plants challenged with *P. grisea*; the purified chitinase had anti-fungal activity against the blast fungus, *P. grisea*, *in vitro*. This study supports our finding of resistance against fungal pathogen in

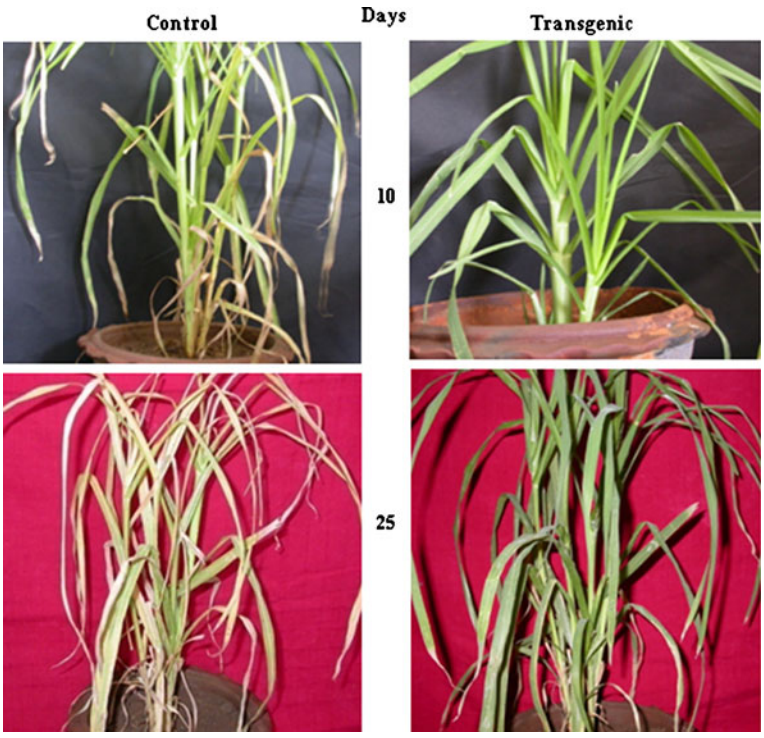

Figure 9. Bioassay of R_1 transgenic plants of GPU 45 (GPU45-19) for leaf blast resistance. The response was captured on 10 and 25 days after spraying the *Pyriculariae grisea* spores.

finger millet plants by overexpression of rice chitinase. Several reports are available on other cereals for increased resistance to fungal pathogens by overexpression of chitinase (Lin *et al.* 1995; Grison *et al.* 1996; Asao *et al.* 1997; Tabei *et al.* 1998; Nishizawa *et al.* 1999; Takatsu *et al.* 1999; Yamamoto *et al.* 2000; Rohini and Rao 2001; Datta *et al.* 2000, 2001; Baisakh *et al.* 2001; Sridevi *et al.* 2003, 2008; Kumar *et al.* 2003; Kalpana *et al.* 2006; Nandakumar *et al.* 2007; Sripriya *et al.* 2008). The plant's resistance to pathogens involves the activation and expression of defense proteins during active defense mechanism, but these mechanisms are too weak or appear too late to be effective for preventing the establishment of pathogens. Hence, increased constitutive expression of introduced chitinase is necessary for arresting the early development of the fungus. Transgenic modification offers a way for the constitutive expression of chitinase gene.

The present study demonstrated a high level of resistance to leaf blast disease by the expression of rice chitinase gene (*chi*11) in finger millet. This study may be

Table 3. Leaf blast assay in R_0 plants of finger millet genotype GPU45 transformed with rice chitinase gene

Plant type	Number of lesions per leaf	Size of lesion (mm)	Disease resistance scale
Non-transformed-1	16.4c	25.4±1.3c	9
Non-transformed-2	15.5c	18.6±1.1b	8
Transgenic line-1	5.8ab	0.2±0.9a	2
Transgenic line-10	6.4ab	0.4±0.8a	3
Transgenic line-12	1.2a	0.1±0.7a	2
Transgenic line-17	1.6a	0.3±1.2a	0
Transgenic line-19	3.2a	0.4±0.9a	2
Transgenic line-24	6.7b	0.4±0.6a	3
Transgenic line-25	4.8ab	0.3±0.7a	3
Transgenic line-29	3.7a	0.1±0.8a	2

Values are mean±SD of three replications. Means followed by a common letter are not significantly different by LSD test (P=0.05 %).

Table 4. Segregation analysis of R_1 finger millet plants transformed with rice chitinase gene

Line number of transgenic plant	Total no. of T_1 seeds	Hygromycin		Ratio	χ^2-value	Probability value (P)
		Resistant	Sensitive			
GPU 45-1	55	41	14	3:1	0.06	>0.60
GPU 45-12	35	27	8	3:1	0.08	>0.60
GPU 45-19	42	32	10	3:1	0.03	>0.60
GPU 45-25	47	36	11	3:1	0.06	>0.60

helpful for inserting other fungal-resistant genes such as glucanase into finger millet by *Agrobacterium*-mediated transformation in future.

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