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RESEARCH ARTICLE

Expression of a bacterial chitinase (*ChiB*) gene enhances resistance against E. polygoni induced powdery mildew disease in the transgenic Black gram (*Vigna mungo* L.) (cv. T9)

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Abstract To enhance the antifungal response of blackgram (*Vigna mungo* L.), transgenic plants were generated by transferring bacterial chitinase gene with a CaMV 35S promoter. The chopped multiple shoot cells developed on the cotyledonary node were transformed by Particle gun method. The calli were raised on the Murashige and Skoog (MS) modified media supplemented with 50mgl^{-1} kanamycin. The transformation efficiency was 13 %. The resultant shoot buds were selected and the antibiotic resistant transgenic plantlets were regenerated. The development of the transgenic plants from the shoot buds took about four to six months. The transgenic status was confirmed by PCR, RT-PCR, Southern and western blot analyses. The transgenic plants exhibited higher chitinase activity than the non-transformed plants. The chitinase activity was examined using the native polyacrylamide in-gel assay. The transgenic plants showed enhanced resistance as evidenced by the delayed onset of the disease and smaller lesions following an in vitro inoculation of the powdery mildew pathogen (E. polygoni DC). The transgenic plants adapted well to the greenhouse and did not show any phenotypic alterations.

Keywords *Vigna mungo* L · Bacterial chitinase · Transgenic plants · Fungal resistance · *E. polygoni*

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Abbreviations

NPT II	Neomycin phosphotransferase
ChiB	Bacterial chitinase
MS	Murashige and Skoog (1962)
BA	N^6-benzyl adenine
NAA	α-naphthalene acetic acid
IBA	Indole3-butyric acid
GUS	β-glucuronidase gene
RT-PCR	Reverse transcriptase polymerase chain reaction
CaMV	Cauliflower mosaic virus
NOS	Nopaline synthase
AA	Ascorbic acid
CA	Citric acid
PPO	Polyphenoloxidase

Introduction

Black gram (*Vigna mungo* L. Hepper) is an important legume. It is a tropical annual herbaceous pulse crop cultivated since the ancient times in India. It is one of the main sources of dietary protein for a majority of population in the developing countries of Asia, Africa and Latin America. It is very nutritious, rich in phosphoric acid and is recommended for diabetics. The seeds have 60 % carbohydrate, 24 % protein and 1–3 % fat on dry weight basis. The productivity of this crop has been greatly limited due to several viral, bacterial and fungal infections, and insect infestation (Singh 1981). There are several varieties of black gram in India (PS-1, Pusa-1, Pusa-2, T9 etc.) but most of these varieties are susceptible to many diseases. The most important among them is powdery mildew caused by *E. polygoni*. This disease may be controlled partially by fungicides. At the same time, environmental safety concerns may also arise and it would, therefore, be imperative to produce pathogen-resistant varieties of the crop.

Organogenesis and plantlet regeneration have been reported in black gram by the culture of multiple shoots developed on cotyledonary node (Das et al. 1998, 2002), cotyledons (Varalaxami et al. 2007), embryonal axes (Acharjee et al. 2012) and leaves (Srivastava and Pandey 2012). The multiple shoots explants are efficient for transformation and regeneration in black gram. The particle gun-mediated transformation has been demonstrated as quite efficient (1.4–22.69 %) in tomato (*Solanum lycopersicum* L.) (Ruma et al. 2009), Castor (*Ricinus communis* L.) (Sailaja et al. 2008), Sugarcane (*Saccharum officinarum* L.) (Kaur et al. 2007) and Cowpea (*Vigna unguiculata* L.) (Ikea et al. 2003). The gene transfer to blackgram has been achieved till date by *Agrobacterium*-mediated transformation (Karthikeyan et al. 1996; Saini et al. 2002; Muraganantham et al. 2007; Saini and Jaiwal 2007; Das 2012), but with low efficiency (1–4.31 %). In order to enhance the transformation efficiency, particle gun bombardment-mediated transformation of legumes is one of the best options. The ballistic device is an alternative method for transformation that overcomes some of the problems associated with the use of *Agrobacterium* in blackgram. An efficient biolistic–mediated genetic transformation of cowpea (*Vigna unguiculata*) and stable Mendelian inheritance of the transgene has already been reported (Ivo et al. 2008).

The bacterial chitinase (*ChiB*) gene has been expressed in the leaves of tobacco using two photosynthetic gene promoters (Jones et al. 1986). This gene has also been expressed in litchi (Das and Rahman 2010). The rice chitinase gene (*Chi11*) has been shown to enhance resistance of bread wheat against herbicide (bialaphos) through biolistics (Chen et al. 1998).

The present work involves the use of Particle gun bombardment-mediated transformation to over express bacterial chitinase gene (*ChiB*) in Blackgram cv. T9. The transgenic fertile plants exhibited higher chitinase activity with increased resistance to powdery mildew disease caused by *E. polygoni* DC.

Materials and methods

Plant materials

Seeds of Black gram cv. T9, obtained from Division of Horticulture, Bihar Agricultural University, Sabour, Bhagalpur, India were washed in running tap water to free them from dust. Then they were dipped into 0.1 % $HgCl_2$ and were kept there for 10 min. After these procedures seeds were rinsed thrice in sterile distilled water under aseptic conditions. The seeds were germinated in MS medium (Murashige and Skoog 1962) supplemented with 2 mgl^{-1} BA. After twelve days of culture, the multiple shoots (six to eight in number) formed at the cotyledonary nodes of these plants (Das et al. 1998) were cut from the base and chopped into small pieces to preculture (for two days) on the MS medium supplemented with 2mgl^{-1} BA followed by transformation.

Transformation of black gram

Particle gun-bombardment method (PDS 1000/He of BioRad) using tungsten particles (microcarrier and size is 0.7–1 μm) sterilized in ethanol suspension was applied for transformation of the target explants. The de-agglomerate particles were suspended in 1 ml of 100 % ethanol in a microtube which was vortexed thrice for 1–2 min. To 50 μl aliquots of tungsten carrier suspension in ethanol, 5 μl of DNA (binary vector pBI121-*ChiB-GUS*, with chitinase and *GUS* genes respectively under the control of 35S constitutive promoter), 50 μl of $CaCl_2$ (2.5 M) and 20 μl of spermidine (0.1 M, free base, tissue culture grade) were mixed in the respective order, while vortexing continuously. After vortexing for 3 more min the microcarriers were spun down at 10,000 rpm for 10 s. The suspension was discarded. The microcarriers were washed with 250 μl of 100 % ethanol by vortexing briefly followed by centrifugation. The supernatant was removed and the microcarrier pellet was resuspended in 60 μl of ethanol. An aliquot (10 μl) of the DNA coated microcarriers was pipetted on to the centre of the macrocarrier holder. The macrocarrier holder without traces of ethanol was mounted on the Particle Gun for bombardment at 1300 psi (psi is pressure per square inch). Multiple shoot explants were arranged in a circle at the centre of a sterile petriplate having regenerable MS medium supplemented with 2 mgl^{-1} BA and 11 % mannitol (as osmoticum which maintains the viability of explants' cell during high pressure of bombardment) and the explants were incubated in the dark at 25 ± 2 °C for two days to allow cell repair and DNA integration. After a period of 16 h following the bombardment, the osmotic pressure of the medium was reduced gradually as described by Kikkert et al. (1996). Following two days of bombardment small aliquots of explants were assayed for transient and stable expression of GUS and the rest was sub-cultured in the fresh regenerable MS medium supplemented with 2 mgl^{-1} BA and 50 mgl^{-1} Kanamycin (antibiotic) following the protocol of Das et al. (2002). The explants were transferred into 100 ml liquid medium consisting of half as strength of MS salts supplemented with MS organics, 0.5 mgl^{-1} BA, 0.1 mgl^{-1} NAA and 3 % sucrose and 50 mgl^{-1} Kanamycin in Erlenmeyer flask of 250 ml agitated at 100 rpm on a rotary shaker (Figs. 1 and 2). The medium was refreshed every third day. Shoot elongation was observed when callus clumps with shoot initials, which were larger than 5 cm^2, were removed from the liquid medium and cultured on one-third strength MS salts supplemented with MS organics, sucrose, 50 mgl^{-1} kanamycin and

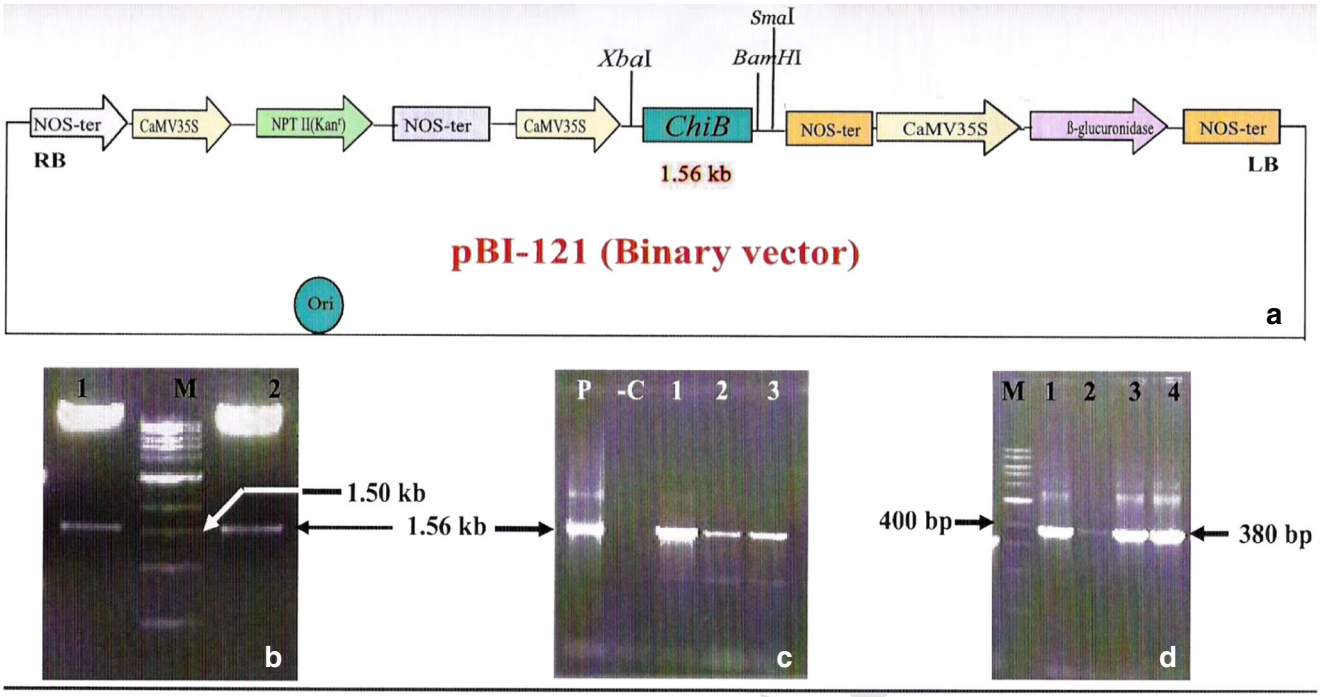

Fig. 1 ChiB, GUS & NPT-II genes under the control of 35S constitutive promoter construct in plasmid pBI 121 and its analysis. **a**. Map of *ChiB* gene constructs. Expression of gene is under the control of CaMV 35 S constitutive promoter. **b**. Enzymatic digestion pattern of recombinant clone by Xba I and Sma I; Lane M: 1 kb DNA ladder, Lanes1 and 2: showing 1.56 kb *ChiB* fragment. **c**. Colony PCR of recombinant clone using gene specified primers. Lane P: recombinant plasmid as a positive control, Lane C: negative control without the recombinant plasmid, Lanes 1, 2 and 3 showing amplicons of 1.56 kb *ChiB* gene. **d**. Colony PCR of recombinant clone using GUS gene specific primers, Lane M: 100 bp DNA ladder, Lanes 1, 2, 3 and 4 showing GUS specific amplicon of 380 bp

0.7 % agar for semi solid medium. For root initiation, elongated shoots (of length greater than 1 cm) were excised and cultured on one–third strength of MS salts supplemented with MS organics, 1 mgl^{-1} IBA and 0.7 % agar semi-solid medium. The rooted plantlets (ca. 5 cm) were transferred into autoclaved vermiculite moistened with Hoagland (Hoagland and Arnon 1950) medium in 6-cm plastic pots and covered with a plastic cover to maintain humidity. Two weeks later, the covers were gradually removed over a period of seven days at high light intensity i.e. 90 µmol m^{-2} s^{-1} and temperature range from 28 to 37 °C for acclimatization, before the plantlets were finally transferred to soil.

Histochemical assay of the transgene (GUS)

For histochemical localization of the GUS activity X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) or X-magenta were used as substrates (Jefferson 1987). Stock of 10 mg/ml was used in which 10 mg of X-gluc was dissolved in 1 ml of dimethylformamide. The working concentration of the X-gluc solution was 1 mg/ml. The dilution of the stock solution to the working solution was done in 50 mM sodium phosphate buffer, pH 7.0. The tissue to be assayed were immersed in 1 mg/ml X-gluc solution and placed at 37 °C for a period of 10 to 12 h. After the incubation time, the samples were placed in 70 % ethanol. Several changes of 70 % ethanol were made for faster discolorations (removal of chlorophyll) of the sample. Once the sample was completely decolorized, they were placed in 30 % sterile glycerol solution in 50 mM sodium phosphate buffer, pH 7.0 and stored at 4 °C.

Polymerase chain reaction (PCR)

To confirm the presence of the bacterial chitinase gene in the transgenic plants, genomic DNA was isolated from 0.5 g of fresh young black gram leaves as described by Lodhi et al. (1994). For the PCR analysis, 200 ng of plant DNA or 4 ng of plasmid DNA was used per 25-µl reaction mixture. The primers were designed to amplify 465 bp fragments of chitinase at 63.6 °C (F5′GCTACTGCTTCAAGGAGGAGAAACA3′; R 5′CTGGTTGTAGCAATCCAGGTTATCG-3′) and 508-bp fragments of NPT-II gene at 52 °C (F-5′ AGCTGCGCCGATGGTTTCTACAA3′; R-5′ ATCGCCTCGCTCCAGTCAATG 3′). The PCR program profile for both the genes was as follows; initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 1.5 min at the annealing temperature of each gene and 1 min at 72 °C, with a final extension at 72 °C for 10 min. The amplified products were run on 1 % agarose gels and visualized by ethidium bromide staining.

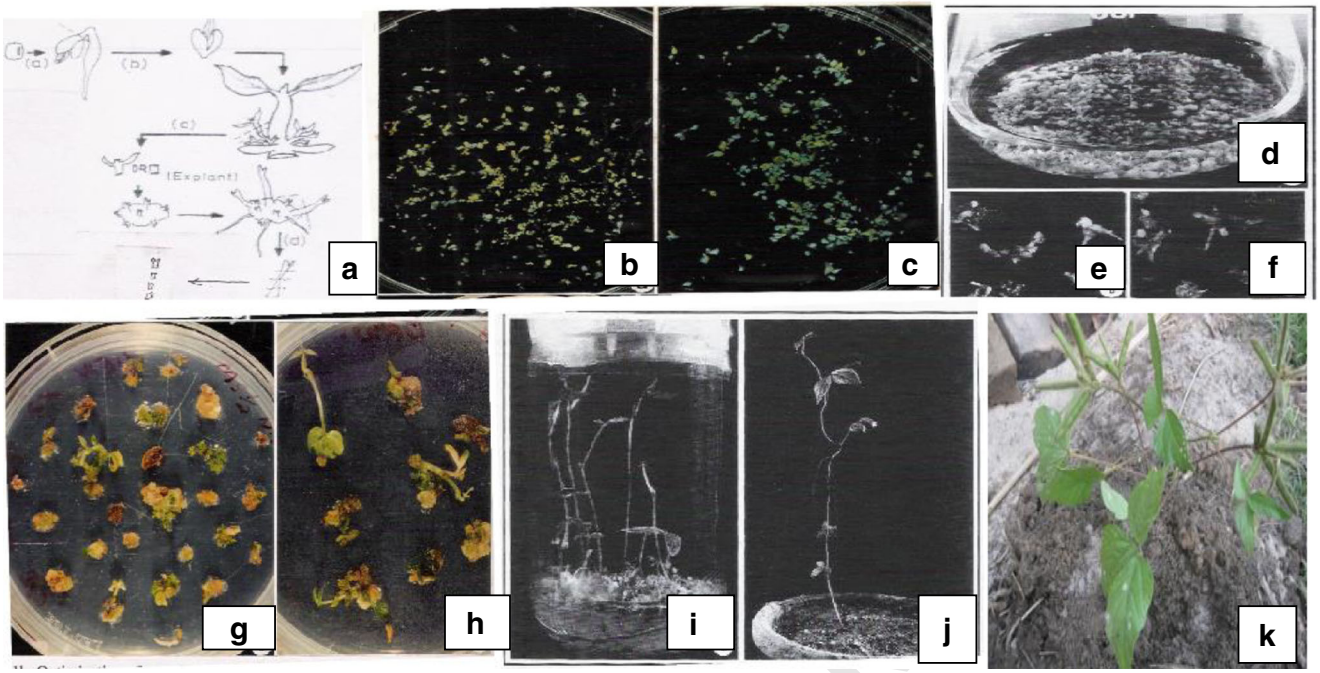

Fig. 2 Production of transgenic black gram plants harboring the bacterial chitinase gene (*ChiB*) by the Particle gun bombardment-mediated transformation method. **a**. Development of multiple shoots on cotyledonary node, excision of multiple shoots from cotyledonary node and chopped into small pieces **b & c** – Transient and long-term expression of transgene–GUS in small pieces of multiple shoots after two days and ninety five days of Particle gun bombardment shows development of blue color in substrate X-gluc reveals the integration of transgene into genome of black gram. **d**, **e& f**- Development of shoot buds on calli of small pieces of explants in liquid culture. **g & h** – Shoots were selected on growing calli on semi-solid culture supplemented with kanamycin antibiotic. **i**- Development of roots on the base of shoots separated from calli and full-grown in vitro plantlet. **j** Transformed plantlets in vermiculite **k**. Transformed fertile plants in field soil bearing pods with seeds

Southern blot analysis

In order to confirm the transgene integration and to determine the number of copies of transgene *(ChiB*) integrated into genomic DNA, Southern blot (Southern 1975) analysis was done. Genomic DNA (10 μg) and plasmid (pBI121-*ChiB-GUS*) as positive control were digested with *Xba* I *or BamH* I (New England Biolab), fragments were separated on 1 % (w/v) agarose gels at 25 V for 16 h. The fractionated DNA was denatured with 0.5 M NaOH for 30 min, neutralized with 25 mM sodium phosphate buffer, pH 6.5 and transferred to positively-charged nylon membranes (Hybond N+, Amersham Biosciences, Hongkong). The transferred DNA was fixed to the membrane by baking at 80 ° C for 30 min followed by UV irradiation (12×10^4 μJ cm^{-2}) using a UV cross-linker (UV-Stratalinker 1800, USA). Blots were hybridized with [α-^{32}P] dCTP-labelled PCR-derived ChiB or NPT-II genes as a probe using random primers labeling kit (New England Biolab) and detection were made by autoradiography carried out on Kodak Xomat X-ray film (Kodak) for one day.

Total RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from leaf tissues using Trizol Reagent as per the manufacturer's instructions (Trizol Reagent, Invitrogen life technologies, San Diego, California, USA). The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometer. To detect the presence of bacterial chitinase mRNA transcripts in the transformants, RT-PCR was carried out. To 5 μg of RNA in a total volume of 12 μl of RNAse free H_2O, 1 μl of 18-mer oligo dT primer was added and the mixture was incubated for 10 min at 70 °C. Then the mRNA was reverse transcribed by addition of 4 μl of RT, 1x of buffer, 1 μl of RNAse inhibitor (40 U/μl) and 2 μl of 5 mM dNTPs. The mixture was incubated for 5 min at 37 °C, and then 1 μl M-MuLV (Moloney Murine Leukemia Virus) Reverse Transcriptase polymerase (200 U/ μl) was added and incubated for 1 h at 42 °C followed by inactivation at 70 °C for 10 min. RT-PCR was carried out on 5 μl of this RT product using *ChiB* gene specific primers at 52 °C (F5′ TCTCCT TCAAGACGGCGTTCTGGTTC-3′;

R5′CTGGTTGTAGCA ATC CAGGTTATC G-3′) and cycle parameter was kept as mentioned in the section earlier to amplify 465-bp of bacterial chitinase. Non-transformed plants cDNA was used as experimental control.

Biochemical analysis

The chitinase levels in the transgenic black gram were determined using colorimetric enzyme assay, in-gel assay and western blot analysis. Total soluble proteins were extracted from frozen leaves (placed at −80 °C for a week) of the transformed and non-transformed samples. Leaves were homogenized with a pestle and mortar in liquid nitrogen and the frozen powder was suspended in 5 volumes of 0.1 M sodium citrate buffer (pH 6.0) containing 20 mM sodium ascorbate and polyclar AT. After two rounds of centrifugation at 13, 000 rpm for 15 min at 4 °C, the supernatants were recovered (Yamamoto et al. 2000). The protein concentrations in the extracts were estimated by the Bradford method (Bradford 1976). Equal amounts (25 μg) of soluble proteins were loaded on 1D SDS-gels and stained with 0.1 % Coomassie brilliant blue R-250 (dissolved in 25 % methanol & 10 % acetic acid mixture for 20 min) dye to ensure equal loading of protein and de-stained in 10 % acetic acid overnight. In western blotting the proteins were transferred into the nitrocellulose membrane and probed with anti-chitinase B antibodies (Generously provided by Dr.M.V.Rajam, Deptt. Of Genetics, South Campus, University of Delhi, India) at 1:5000 dilution. The specific position of antigen-antibody complex on the membrane was visualized by using alkaline phosphatase linked to secondary antibodies. In-gel assay of chitinase: Chitinases isoforms were visualized by glycol chitin SDS-PAGE. The extract containing 70–100 μg of soluble protein from the different transgenic lines as well as the Non-transformed control was diluted (1:10) and subjected to SDS-PAGE with 5 % stacking gels and 12 % acrylamide resolving gels (Laemmli 1970) incorporated with 0.01 % glycol chitin as described by Trudel and Asselin (1989, 1990). Glycol chitin was prepared by acetylation of glycol chitosan, where 1 g of glycol chitosan (G-7753, Sigma, USA) was dissolved in 20 ml of 10 % acetic acid and the viscous solution was mixed and kept overnight at room temperature, and then 90 ml methanol was added and mixed continuously. The solution was filtered through Whatman filter paper (No.3) under vacuum. The resulting filtrate was mixed with 1.5 ml acetate anhydride. The formed gel was kept at room temperature for 30 min, the excessive liquid was poured off, and the gel was cut into small slices and homogenized by homogenizer. The homogenate was centrifuged at room temperature at 13,000 rpm for 15 min; the pellet was re-suspended in 100 ml of double distilled water so that the final concentration of that stock was 1 %. After electrophoresis, the gel was incubated with Triton X-100 solution, consisting of 0.1 M NaOAc (pH 5.0) and 1 % Triton X-100, at 37 ° C for 2 h. The resultant gel with Rainbow marker was then stained with Coomassie Brilliant Blue R-250 for 1 h and de-stained with double distilled water for overnight. Gels were photographed with a Kodak digital camera and contrast was enhanced using Adobe Photoshop version (7.0) software.

Quantitative assay of Chitinase enzyme activity A solubilized, ethylene glycol-chitin (Sigma-Aldrich) was used as a substrate for chitinase activity assay. The colorimetric analysis of chitinase enzyme activity of PCR, Southern and RT-PCR positive transgenic plants was done following the protocol of Stephan and Wolf (1990) with slight modifications. The aliquots of 300 μl of ethylene glycol-chitin (stock 2 mg/ml) were mixed with 100 μl of 200 mM sodium acetate buffer, pH 5.0 and 0.5 ml enzyme solution, and then incubated for 60 min. at 37 °C in the circulating water bath. The reaction was terminated by the addition of 100 μl HCl (1.0 N) on ice and incubated for 10 min. to facilitate precipitation of the non-degraded substrate (chitin). It was then centrifuged at 14,000 rpm for 5 min. The resulting N-acetyl glucosamine (GlcNAc) residues were colorimetrically measured by the dinitrosalicylic acid (DNSA) method (Miller 1959). For this to 1 ml of the reaction mixture, 1 ml of DNSA was added and boiled for 10 min. and then 0.4 ml of Potassium-Sodium tartarate was added. The mixture was cooled at room temperature and OD was taken at 540 nm. As appropriate controls, enzyme and substrate blanks were included in the experiment. One unit was defined as the amount of enzyme that produced 1 μmol of reducing sugars corresponding to *N*-acetyl-D-glucosamine in 1 min.

Pathogenicity test against powdery mildew

Tolerance potential of the transgenic black gram carrying bacterial chitinase gene was evaluated against powdery mildew caused by E. polygoni. The developing secondary or tertiary leaves were detached from the in vitro grown transgenic plants. Two leaves from each transgenic plant were placed adaxial side up onto 0.6 % (w/v) agar containing 40 mgl^{-1} benzodiazoloe in a Petri dish. As control, leaves were taken from non-transformed regenerated plants. Since E. polygoni is an obligate ectophytic parasite, so it can't be cultured on an artificial medium (Srivastava 2004) but it can be cultured on leaf disc in water (Morrison 1960). The spores were collected in the aqueous washing (having 0.01 % (v/v) Tween 20) of infected leaves obtained from Horticulture department, Bihar Agricultural University, Sabour, Bhagalpur. The spore suspension, 0.5 ml (10^6 spores/ml) was sprayed on to each Petri dish containing moistened leaf and kept at saturated humidity at 25 °C. The degree of disease severity was scored using a visual assessment scale based on the size and characteristics of necrotic lesions. A 5-point disease rating scale based on the approximate percentage of leaf necrotic area after 15–28 days

of inoculation (1 = 0 %; 2 = 1–20 %; 2 = 20–30 %; 3 = 30–40 %; 4 = 40–50 %; 5 = >50 %) (Yamamoto et al. 2000; Jayaraj and Punja 2007) was employed. The number of days required for the onset and complete chlorosis in each leaf in comparison to control (Fig. 7a–d) was recorded. The diseased leaves were digitally photographed. A portion of leaves of both non-transgenic and transgenic plants were electron micro-graphed (Fig. 7e) which showed that pathogenic fungal spores could easily germinate, ramify mycelia and also invade the leaf surface cells of the non transgenic regenerated black gram plants. These leaves developed powdery mildew disease. In the transgenic plants, pathogenic spores germinated well but mycelial growth was stunted leading to suppression of the disease.

Electron micrography

For surface ultrastructure study, small (1–8 mm) leaves of both transgenic and non-transgenic black gram (*Vigna mungo* L.) 'plants were sprayed' with pathogenic fungal conidia/spores' solution and left for 2–4 days. For scanning electron microscope (SEM), the specimen is normally vacuum dried. Fixation of the black gram leaves was performed by incubation in a solution of a buffered chemical fixative, such as 2.5 % glutaraldehyde in combination with 2 % formaldehyde in 0.1 M phosphate buffer at pH 7.2 containing 0.03 M sucrose for overnight at 4 °C. It was subsequently washed in 0.1 M phosphate buffer with 0.3 M sucrose for 1 h. The samples were then dehydrated in an acetone series (30–100 %). Drying was achieved till the critical point drying (CPD). For this, liquid carbon dioxide was converted into gas by raising the temperature of the CPD apparatus. To remove CO_2 gas, the CPD exhaust was opened to release pressure of the CPD apparatus. The mounted specimen was coated with the gold particles with the help of sputter coater and examined at 20 KV under the SEM model- Leo VP 635 (Zeiss). Electron microscopic studies were done separately for both transformed and non-transformed detached leaves of black gram plants (Fig. 7e a&b).

An average of each disease value was taken in triplicates. In each experiment 6 detached leaves were taken in each Petridish. The disease values were rated based on the approximate percentage of leaf necrotic area after 15–28 days of inoculation. The percentage response of disease rating scale was different in different black gram plant leaves. The statistical significance on the disease value was calculated by one-way ANOVA followed by Tukey's multiple comparison tests. All data analyses were performed using the Graph Pad software (Graph Pad in Stat. Software Inc. San Diego, CA 92130, USA).

Molecular analysis in T1 transgenic plants of blackgram (*Vigna mungo* L)

Seeds collected from T_0 fertile transgenic plants were germinated in the field soil and divided into seven parts. 1. The hypocotyl portion of germinated seedlings was inoculated after sterilization on MS medium supplemented with $2mgl^{-1}$ BAP for the development of callus in vitro condition under standard tissue culture condition and this callus was put in X-gluc solution for GUS assay as mentioned in Meterials & methods (MM) 2. The germinated seedlings were put in field soil and added nutrients regularly into the field soil for proper growth of transgenic black gram plants in natural condition '(T1generation)'. The leaves were harvested; isolated genomic DNA both for GUS and bacterial chitinase genes were amplified through PCR and Southern hybridization was done as mentioned in MM. 3. The RNA was isolated from leaf tissue and Northern hybridization was done as mentioned in MM. 4. The protein was isolated from leaves extract and Western hybridization was done as mentioned in MM 5. In-gel assay determines the isomeric forms of bacterial chitinase protein .6. Bacterial chitinase activity was assayed from leaves of transgenic plant having bacterial chitinase gene as mentioned in MM. 7. The pathogenic fungal spores which cause powdery mildew disease were sprayed both in transgenic (T_1) and non-transgenic plant.

Results

Characterization of the transgenic black gram

PCR analysis

Existence of the pBI121-ChiB gene in the regenerated black gram plantlets was examined by PCR analysis using gene-specific primers that generated a 465 bp fragment. The NPT-II (neomycin phosphotransferase) gene was also detected by PCR with gene specific primers (F-5′ AGCTGCGCCGATGGTTTCTACAA3′, R-5′ ATCGCCTCGCTCCAGTCAATG3′) that gave rise to a fragment 508 bp. All nine transformants (B-Chi1, 2, 4, 5, 9, 10, 14, 15, and 18) were positive for the 508-bp NPT-II band (Fig. 3a) but only five (B-Chi 1, 4, 9, 15 and 18) were found to possess 465-bp (Fig. 3b) bacterial chitinase gene. There was no amplification observed in the non-transformed plant. For further analysis only these five plants were used.

The foreign genes and their copy number pattern in the nuclear genome of the PCR positive transgenic lines were shown by Southern hybridization. The *Xba* I and 'BamH I' fragments were released as the *ChiB* gene cassette (~1.56 kb). The blot probed with ^{32}P-dCTP labeled *ChiB* cDNA in all the five transgenic lines showed ~1.56 kb band as expected. The

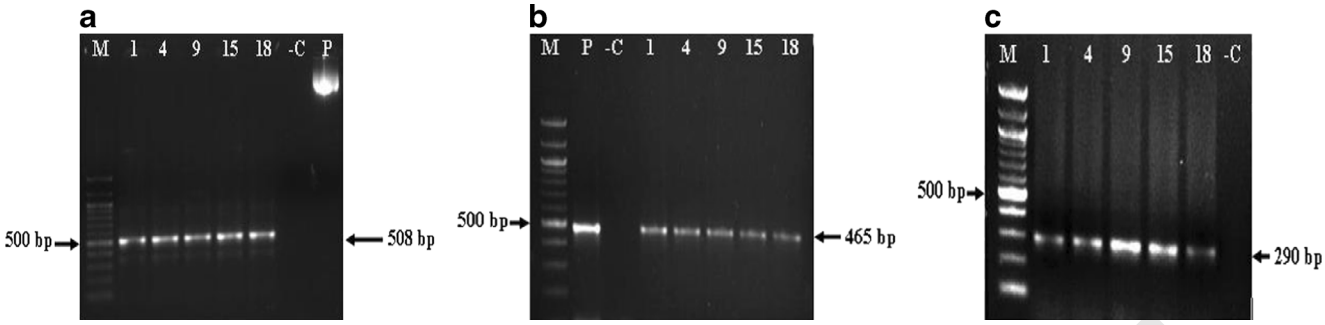

Fig. 3 PCR analysis of plant lines for transgenes (**a**- NPT-II gene), (**b**- *ChiB* gene). 200 ng of plant genomic DNA was used for each reaction. *Numbers* indicate plant lines (1, 4, 9, 15 and 18), *P*- Plasmid positive control; −C- untransformed plant negative control; *M*- 1 Kb marker; *Arrow* indicates amplicon size. **c**. RT-PCR analysis of plant lines for transgenes. 400 ng of total RNA was used for each reaction. *Numbers* indicate plant lines (1, 4, 9, 15 and 18); −C- non-transformed plant negative control; *M*- 100 bp marker; *Arrow* indicates amplicon size

genomic DNA was also digested with *Sac* II, the lone restriction site on the T-DNA region, probed with ^{32}P-dCTP labeled NPT-II gene fragment. Single band appeared suggesting single copy integration in all five lines (Fig. 4a and b).

RT-PCR analysis was used to check the expression of *ChiB* gene. Total RNA was taken from all five samples and RT-PCR analysis was performed using primers specific for the mRNA sequence of *ChiB*. A 465-bp amplified fragment of the *ChiB* transcript confirmed the expression of the bacterial chitinase gene. No amplification was observed in the RNA samples isolated from the un-transformed plant (Fig. 3c).

In-gel assay analysis of proteins of the transformed and non-transformed plants resolved on the SDS-PAGE showed a number of chitinase isoforms (Fig. 5). Non-transformed plants extract displayed chitinase isoforms of molecular weights at 21 and 30 kDa. But an additional isoform of 35 kDa was seen only in the transformed lines, as expected.

Western blot analysis of the representative lines employing polyclonal antibodies raised against bacterial chitinase showed presence of a single prominent band corresponding to the size of 35 kDa indicating its robust expression (Fig. 6c). Higher chitinase activity in all the transgenic plants (chi4, chi9, chi15 and chi18) than in the non-transgenic ones (Table 1) was quite obvious. Lines 4 and 15 showed approximately two and three fold more increase in the enzyme activity than the non-transgenic ones, while lines 9 and 18 showed approximately one and half fold increase in the activity (Figs. 6 and 7)

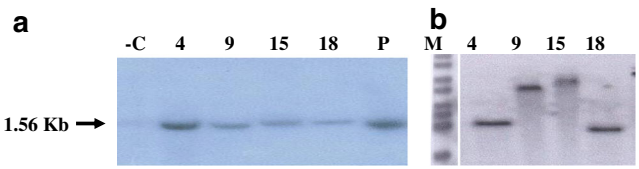

Fig. 4 Southern Blot analysis of transgenic black gram cv T9. Genomic DNA (5 μg) and plasmid digested with *Xba* I (**a**) or BamH I (**b**) were probed with PCR-generated fragments of chitinase (*ChiB*) and NPT-II gene. (*Lanes* – *C*- non-transformed plant, *P*- plasmid- pBI121-ChiB, *4, 9, 15, 18*- different transgenic lines, *M*- 1 Kb marker)

Evaluation of powdery mildew resistance in the transgenic black gram

The detached leaves of the transgenic plants were tested for resistance to the obligate foliar ectoparasitic fungus, E. polygoni. Both Chi-4 and Chi-15 lines showed disease rating scores of 2.0 and 1.3 as an average score of three experiments, respectively, as compared to a score of 4.7 for the non-transformant (Table 1). These results indicated that the two transformants exhibited partial resistance to E. polygoni because there was delay in the spread of lesion areas of the disease. The degree of disease symptoms correlated well with the level of chitinase enzyme activity.

The number of days required for the onset of necrosis was also studied (Table 1). Corresponding to the results on disease index, both Chi-4 and Chi-15 lines took longer period for the necrosis to develop and completely cover the whole leaf area. However, with longer durations all leaves succumbed to the disease. Besides the detached leaves, pathogenicity of E. polygoni on leaves of both transgenic and non-transgenic plants was also studied in the intact plants. It was found that the spores were able to germinate but unable to develop mycelia and produce disease symptoms on the leaves of the transgenic plant. The spores, however, germinated very fast,

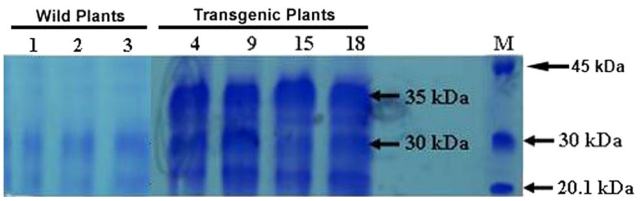

Fig. 5 Biochemical analysis of transformed and non-transformed black gram plants (cv T9). Detection of chitinolytic activity of chitinase after glycol chitin SDS-PAGE electrophoresis of untransformed and transformed black gram plants cv T9. (Lanes- 1, 2 and 3 is non-transformed plants showing two chitinase isoforms of 21 and 30 kDa. Lanes- 4, 9, 15, and 18 are individual transformed lines showing three isoforms of chitinsae i e. 21 kDa and 30 kDa like normal non-transformed plants and 35 kDa of unique size translated from bacterial chitinase (*ChiB*) gene. M-Rainbow marker was used as protein molecular weight standard

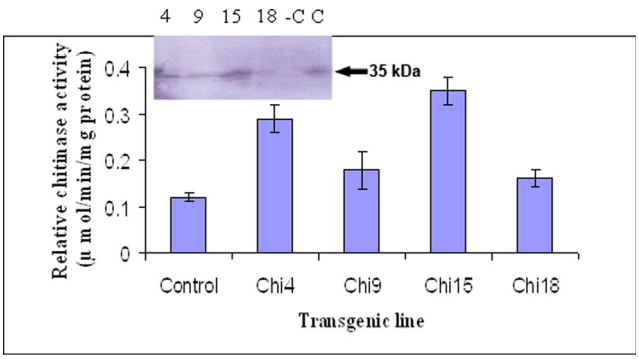

Fig. 6 Chitinase enzyme assay of different transgenic lines of black gram cv. T9. Average values of three different experiments each in duplicates were plotted in the graphs. Inset shows Western blots analysis showing 35 kDa bands. Chitinase activity (1 µmol/min/mgprotein): Control (0.12), Transgenic: Chi 4 (0.29), Chi 9 (0.19), Chi15 (0, 35) and Chi18 (0.18)

developed mycelia and symptoms of disease (as white powdery patches) on the leaves of the non- transformed plant by 8–18 days. Other green parts later became dull colored. These patches gradually increased in size and covered both surfaces of leaves in 20–69 days. In severe infection, foliage became whitish yellow causing premature defoliation. These symptoms were significantly resisted in the transgenic lines.

Molecular analysis of T_1 transgenic Blackgram

The transgenic plants obtained were GUS positive by histochemical assay and PCR positive with the bacterial chitinase, NPT-II and GUS genes with specific primers as checked in different experiments. The PCR positive were subjected to Southern and western blot analysis. Presence of one copy of the bacterial chitinase gene in the transgenics through biolistics showed stable integration. Stability of the integrated gene was proven by analyzing T_0 & T_1 generation of the transgene. In the western blot analysis, a 35 kDa band corresponded to the expression of the bacterial chitinase protein in the transgene. In gel assay confirmed the isoforms (21, 30 and 35 kDa) of chitinase protein and 35 kDa was only found in transgenic plants. Pathogenicity experiments by spraying of pathogenic spore's solution of E. polygoni on transgenic and non-transgenic plants, it was observed that no mycelium formation and consequently no development of disease in transgenic plant but fully development of disease and leaves were perished and abscised in non transgenic plants. All these experiments showed improved fungal tolerance in transgenic plants in both T_0 & T_1 generation (Fig. 8a and b).

Table 1 Tolerant potential of transgenic black gram plants to E. polygoni and number of days required for onset of disease and the complete leaf necrosis on detached leaves from bacterial chitinase transgenic lines

Lines	Disease rating	First symptom (days)	Fully covered
B -Chi-1	4.6 ± 0.52	10	20
B -Chi-4	2.0 ± 0.55	15	64
B -Chi-9	3.5 ± 0.46	12	22
B -Chi-15	1.3 ± 0.58	18	69
B-Chi-18	4.0 ± 0.49	10	29
Non-transformed	4.7 ± 0.51	8	14

Discussion

In this study multiple transformed shoots were developed on the cotyledonary nodes by Particle gun bombardment method. The transformation process induced cellular necrosis to some extent, but recovered soon following special treatments (Vidal et al. 2003). Tissue preculture in MSB2 medium, prior to bombardment yielded better frequency of transformation (Barcelo et al. 1994). This stress possibly causes browning of the tissue due to *de novo* generation of PPO oxidized polyphenols from the vacuole (Lagrimi 1992). To check this process antioxidants like AA and CA (250 mgl^{-1} of each) were used in the liquid culture. Large number of shoot buds in the MS medium (Das et al. 2002) were formed. A few of these shoot buds on calli elongated and the rest necrosed and perished on subsequent transfer to semisolid medium. Necrosis could be due to localization of high concentration of antibiotics and less formation of escapes in the semi solid medium. The transformation efficiency was approx. 13 % that may be attributed to strong physical force of bombardment with the gene gun. These shoot buds rooted well and developed in robust formed sturdy rooted plantlets. Land transfer of the plantlets was 90 % successful without somaclonal abnormalities.

Molecular analyses demonstrated successful integration of T-DNA into the plant genomic DNA. PCR and Southern blot experiments demonstrated complete insertion of the entire fragment into the genomic DNA in toto. Five transformants (Chi 1, 4, 9, 15 and 18) exhibited clear bands in Southern blot when probed with the *ChiB* gene. RT-PCR and restriction analyses further confirmed presence of the active form of the putative gene in the transformed plant genome. Integrated density value (IDV) assessment of RT-PCR products showed that Chi15 as the best of all transformants in terms of expression of *ChiB*.

Expression of the *ChiB* as shown by Western blotting proved that it is physiologically functional. Based on in-gel assay, two regular chitinase isoforms in the leaves of the non-transformed and the transformed *Vigna mungo* L. cv. T9 plants were observed (Fig. 5). An additional 35 kDa chitinase band was observed only in the transformed lines indicating that the foreign gene was indeed expressed. Earlier (Mohammadi and Karr 2002) four isoforms of chitinase was found in soybean nodules. It is possible that different chitinase isoforms are effective against different fungal pathogens

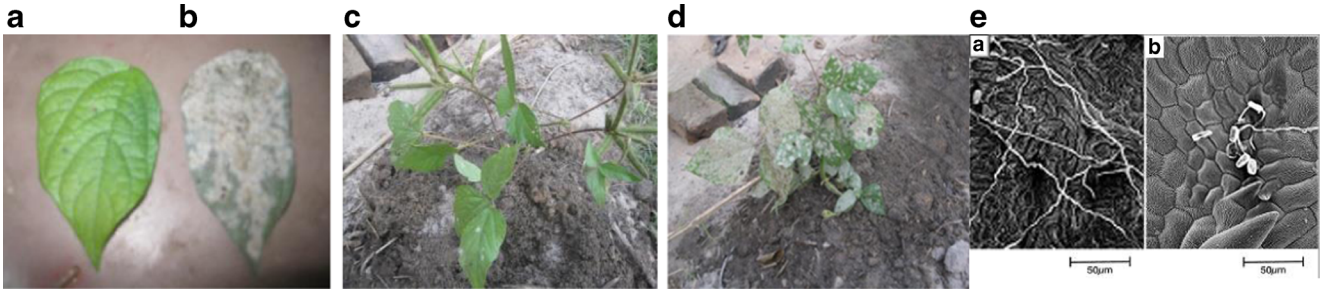

Fig. 7 Evaluation of pathogenicity of E. polygoni both on transgenic and non-transgenic detached leaves of black gram plants **a** Transgenic black gram cv T9 leaf showing spores of E. polygoni couldn't grow and consequently no development of disease. **b**. Non-transformed black gram cv T9 leaf showing growth of spores of pathogen E. polygoni on leaf surface and development of powdery mildew disease. **c** Transformed black gram with high yield **d**. Non-transformed black gram with very low yield **e**. Electron micrographs of non-transformed and transformed black gram leaves' showing the growth of mycelium of pathogen (E. polygoni.) *a*, Electron micrograph of non-transformed black gram plant's leaf showing germination of fungal spores and growth of mycelia and entering inside leaf cells *b*. Electron micrograph of transformed black gram plant's leaf showing very small germination of fungal spores and no growth of mycelia

(Giannakis et al. 1998). During the evolution of biochemical defense systems these isoforms were apparently evolved by soybean plant to resist fungal infection of different kinds.

The transgenic line Chi-15 showed approximately two fold more increase in the chitinase enzyme activity while the rest (Chi-1, 4, 9 and 18) showed approximately 1.5 fold increase only. This is correlated well with the degree of resistance to the pathogens. The statistical correlation coefficient between chitinase activity and disease rating scale is −0.1726. Thus the chitinase activity of the transgenic plants increases with corresponding reduction in the disease rating scale. Previous reports in the transgenic canola (Broglie et al. 1991), strawberry (Asao et al. 1997), rice (Nishizawa et al. 1999), tobacco (Broglie et al. 1991) and cotton (Emani et al. 2003) adequately support the present observations. Delays in the development of symptoms of fungal disease in these transgenic plants expressing were closely comparable to the observations of transgenic plants expressing chitinase gene Trudel and Asselin 1990; Asao et al. 1997 and Yamamoto et al. 2000. Yamamoto et al. (2000) produced transgenic grape plants expressing the rice chitinase gene showing significant resistance against *Uncinula necator* infection, but not so significant against *Elisinoe ampelina*. Similarly, very high inoculation densities of leaf rust spores led to the same severity of symptoms in the transgenic wheat plants expressing an integrated *chitinase* gene as in the control plants (Oldach et al. 2001). These observations may be attributed to non-expression of the chitinase gene isoform that would specifically control pathological effects of the mentioned pathogens. Similar datas of GUS, PCR, Southern, Northern, Western blotting, in gel assay showed different isoforms of Chitinase protein, chitinase activity and pathogenicity were found similarly in both T_0 and T_1 generations showed fungal tolerance in transgenic plants confirmed the stable integration of bacterial chitinase into nuclear genome.

In vitro inoculation method was followed using detached leaves under controlled conditions to evaluate the resistance against the powdery mildew disease in the blackgram transgenic plants. Significant resistance by the transgenic plant leaves (at least till the 18th day) as compared to the controls was observed. Beyond this period resistance afforded by the transgene started waning. It may be due to non-availability of necessary ingredients to the detached leaves for survival and resistance from the mother plant. However, for eighteen days these leaves resisted against the pathogen entirely on their own.

Briefly, the successful transformation of multiple shoots derived from the cotyledonary nodes of *Vigna mungo* L.cv. T9 were regenerated into rooted plants. Successful integration of the bacterial chitinase gene into the black gram genome showed significant resistance to powdery mildew disease not only on the rooted plant leaves but also on the detached leaves. These findings suggest that the bacterial chitinase *ChiB* gene could be utilized as a genetic source of disease control for breeding and improving crop plants.

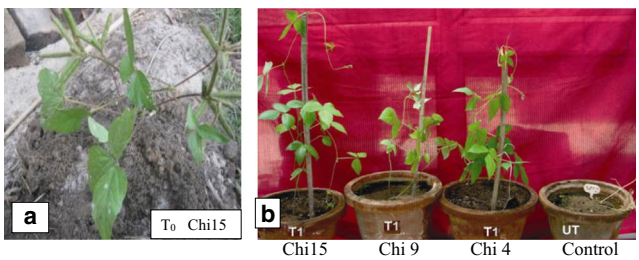

Fig. 8 A and B generated transgenic black gram plants. A. is T0 generated plants having legume fruits. B is three transgenic lines i.e. (i) T_1 (Chi 15), (ii) T_1 (chi 4), (iii) T_1 (Chi 9) generated transgenic plants from T_0 seeds with three lines i.e. Chi15 and Chi 9 have high chitinase activity than the chi 4 and UT is untreated or non-transgenic plants

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