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Transgenic *indica* Rice Variety Pusa Basmati 1 Constitutively Expressing a Rice Chitinase Gene Exhibits Enhanced Resistance to *Rhizoctonia solani*

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***Agrobacterium*-mediated transformation of an elite *indica* rice variety, Pusa Basmati 1, was performed using LBA4404 (pSB1, pMKU-RF2) that harbours a rice chitinase gene (*chi*11) under the control of the maize ubiquitin (*Ubi*1) promoter-intron. Right border (*gus*) and left border (*hph*) flanking sequences and the transgene (*chi*11) in the middle of the T-DNA were used as probes in Southern analysis. Out of eleven independent T_0 plants regenerated, three had single copy T-DNA insertions and eight had multiple T-DNA insertions. Nine T_0 plants carried the complete T-DNA with the chitinase transgene. Two T_0 plants did not carry *chi*11, though they had other T-DNA portions. Three plants harbouring single copy insertions and one plant harbouring two inserted copies were analyzed in detail. A segregation ratio of 3:1, reflecting T-DNA insertion at a single locus, was observed in the progeny of all the four T_0 plants. Northern and western blot analyses of T_1 plants revealed constitutive expression of chitinase at high levels. Bioassays of T_1 plants indicated enhanced resistance to the sheath blight pathogen, *Rhizoctonia solani*, in comparison to control plants. A homozygous transgenic line was established from one T_0 line, which exhibited the maximum resistance to *R. solani*.**

***Key words*:** sheath blight, *Rhizoctonia solani*, chitinase, transgenic rice, *Agrobacterium.*

Sheath blight disease, caused by *Rhizoctonia solani*, is a major fungal disease of rice. Cultivars resistant to this disease have not been identified so far (1). Genetic engineering of rice using antifungal genes offers an opportunity to develop sheath blight resistance in rice. An important plant defense mechanism against pathogen attack is the synthesis of 'pathogenesis-related' (PR) proteins. The PR proteins are of host origin and are synthesized under specific pathological and stress conditions (2, 3). Chitinase (PR-3) has been extensively studied for its antifungal role (4).

The usefulness of constitutive expression of chitinase in transgenic plants for developing fungal resistance has been evaluated in several plants. Broglie *et al* (5) first showed enhanced resistance to *R. solani* in transgenic tobacco and canola plants harbouring a bean chitinase gene under the control of the CaMV 35S promoter. Tobacco chitinase was found to be effective against *Cercospora arachidicola* in peanut (6) and against *Botrytis cinerea*, *R. solani* and *Sclerotium rolfsii* in carrot (7). Tomato chitinase gene expressed in canola developed resistance against *Cylindrosporium concentricum* and *Sclerotinia sclerotiorum* (8). Transgenic tomato expressing *Lycopersicon chilense* chitinase gene exhibited resistance to *Verticillium dahliae* races 1 and 2 (9). Barley chitinase expression in wheat conferred resistance to *Blumeria graminis* and *Puccinia recondita* (10). In addition, chitinases from fungi like *Rhizopus oligosporus* (11) and *Trichoderma harzianum* (12) also enhanced the resistance levels in transgenic tobacco. Chitinase expression did not contribute to fungal resistance in certain cases. Tobacco chitinase expression in carrot failed to reduce the infection caused by *Thielaviopsis basicola* and *Alternaria radicina* (7) and in tobacco against *Cercospora nicotianae* (13). Similarly, expression of sugarbeet chitinase in tobacco did not lead to resistance against *Cercospora nicotianae* (14).

Over-expression of rice chitinase has been explored in many plants to develop resistance to fungal pathogens.

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Abbreviations: AS, acetosyringone; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; *chi*11, rice chitinase gene; CI, callus-induction; 2,4-D, 2,4-dichlorophenoxyacetic acid; *gus*, β-glucuronidase; *hyg*, hygromycin; *hph*, hygromycin phosphotransferase; *int-gus*, intron-containing *gus* gene; LB, left T-DNA border; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; NBT, nitroblue tetrazolium, PB1, Pusa Basmati 1; PR, pathogenesis-related; RM, regeneration medium; RB, right T-DNA border; TTBS, Tween-Tris buffered saline; *Ubi*1, maize ubiquitin promoter-intron.

Transgenic rice plants obtained by PEG-mediated transformation of *chi*11 exhibited high levels of chitinase expression and enhanced resistance to *R. solani* (15). *Agrobacterium*-mediated transformation of *indica* rice genotypes (Basmati 122, Tulsi and Vaidehi) using *chi*11 also restricted the growth of *R. solani* (16). Datta *et al* (17) later showed that the infection-related rice chitinase gene (*RC*7), when introduced into cultivars like IR72, IR64 and Chinsurah Boro II by biolistic and PEG-mediated transformation, exhibited increased tolerance to *R. solani*. Transgenic rice generated by *Agrobacterium*-mediated transformation of rice chitinase genes (*cht*-2 and *cht*-3) showed greater retardation in blast symptoms and to a reduction in lesion number and size (18). The above strategy was extended to cucumber (19, 20), rose (21) and grapes (22), to develop resistance to the respective fungal pathogens.

In this study, we report a high frequency of *Agrobacterium*-mediated transformation of an elite *indica* rice variety Pusa Basmati 1 with rice chitinase gene (*chi*11) driven by *Ubi*1 promoter-intron. Integration of T-DNA was confirmed in eleven independent transgenic lines. Copy numbers of T-DNA insertions were determined using right and left border probes. Detailed Southern analysis using probes derived from the left border, right border and middle regions of the T-DNA helped in confirming the transfer of single copies of complete T-DNA. Inheritance of the transgene in T_1 plants was demonstrated. Bioassay of T_1 plants of different transgenic lines with *R. solani* showed varying levels of resistance in comparison with controls. A homozygous line was developed from a transgenic line that exhibited highest level of resistance.

Materials and Methods

Binary vector and Agrobacterium strain — A 3.1-kb *Hin*dIII fragment carrying the rice chitinase gene (*chi*11) (23) driven by maize *Ubi*1 promoter-intron was subcloned into pCAMBIA1301 (CAMBIA MGRS Accession number TG148) obtained from the Center for Application of Molecular Biology to International Agriculture (CAMBIA), Canberra, Australia. The resultant binary vector, pMKU-RF2, contains hygromycin phosphotransferase (*hph*) gene as plant selection marker and an intron-containing *gus* gene (*int-gus*) as a reporter gene (*see* Fig. 1). The binary vector was mobilized into the *Agrobacterium vir* helper strain LBA4404 harbouring a plasmid, pSB1 (24) by triparental mating (25). The resultant transconjugant was confirmed by Southern analysis (26).

Plant material and callus initiation — Mature seeds of *Oryza sativa* L subsp *indica* variety Pusa Basmati 1 (PB1) were dehusked and surface-sterilized as described by Vijayachandra *et al* (27). Sterilized seeds were placed on a callus-induction (CI) medium [MS medium (28) with the following supplements: proline (500 mg l^{-1}), 2,4-D (2.5 mg l^{-1}), casein hydrolysate (300 mg l^{-1}), sucrose (30 g l^{-1}) and Phytagel (2.25 g l^{-1}), pH 5.8]. After 21 days incubation in dark at 25°C, the scutellum-derived calli were subcultured and preincubated on CI medium for four days.

Agrobacterium-mediated transformation of PB1 — *Agrobacterium tumefaciens* LBA4404 (pSB1, pMKU-RF2) was grown in AB minimal medium (29) at 28 °C in a shaker (200 rpm) until an optical density of 1.0 (A_{600}) was reached. Infection and co-cultivation were carried out as described by Rashid *et al* (30) with some modifications. Bacteria were centrifuged at 1,100 x g for 10 min and resuspended with an equal amount of AA medium (31) supplemented with 50 μM acetosyringone (AS). The calli were soaked in the bacterial suspension and swirled gently for 15 min. Infected calli were blot-dried and placed on Whatman No.1 paper kept over the co-cultivation medium [CI medium supplemented with glucose (10 g l^{-1}), Phytagel (3 g l^{-1}) and 100 μM AS] for three days in dark.

The co-cultivated calli were rinsed twice in liquid CI medium, rinsed once in CI medium supplemented with hygromycin (50 mg l^{-1}) and cefotaxime (250 mg l^{-1}) and placed on the selection medium [CI medium supplemented with Phytagel (4 g l^{-1}), hygromycin (50 mg l^{-1}) and cefotaxime (250 mg l^{-1})] for 14 days. After three cycles of selection (21 days per cycle), calli were analyzed for GUS activity as described by Hiei *et al* (32).

GUS-positive, stably transformed calli were transferred to regeneration (RM) medium [MS medium supplemented with kinetin (3 mg l^{-1}), NAA (1.5 mg l^{-1}), Phytagel (6 g l^{-1}), hygromycin (40 mg l^{-1}) and cefotaxime (250 mg l^{-1})]. After 14 days of incubation in dark, the calli were placed in light for shoot development. The shoots were transferred to rooting medium [½ MS basal medium supplemented with Phytagel (3 g l^{-1}), hygromycin (40 mg l^{-1}) and cefotaxime (250 mg l^{-1})]. Portions of leaves and roots were analyzed for GUS activity. Regenerated GUS-positive plantlets were acclimatized in clay soil and grown in a transgenic greenhouse.

DNA analysis — DNA was extracted from control and transformed plants (33) and estimated using Hoechst dye 33258 in a DNA Fluorometer. Ten microgram samples of DNA from control and transformed plants were digested with *Hin*d III and separated in 1% agarose gels. DNA was transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA) for Southern hybridization analysis (26). A right border (RB)-flanking 1.8-kb *gus* coding sequence, a left border (LB)-flanking 1.1-kb *hph* coding sequence and the 1.1-kb *chi*11 from the middle of the T-DNA (*see* Fig. 1) were labelled with [α-^{32}P]dCTP using the Megaprime™ DNA labelling system (Amersham Pharmacia Biotech, Little Chalfont, England). [α-^{32}P]dCTP was bought from BRIT (Mumbai, India).

Segregation analysis — Seeds from selfed T_0 plants were surface-sterilized and germinated on ½ MS basal medium (supplemented with 0.8% agar) in dark for three days. The germinated seedlings were transferred to the same medium with 50 mg l^{-1} hygromycin and placed in light. Hygromycin-resistant (Hyg^R) and -sensitive (Hyg^S) plants were scored after 10 days. χ^2 test was performed to validate the data for 3:1 segregation ratio.

Northern analysis — Total RNA was extracted from T_1 plants as described by Pawlowski *et al* (34) and quantified at 260 nm using a spectrophotometer. Ten microgram samples of total RNA from control and transgenic T_1 plants were separated in a denaturing 1.5% agarose gel containing 1% formaldehyde. The presence of equal amounts of RNA in each lane was confirmed by staining the gel with ethidium bromide. Formaldehyde was eluted from the gel using an elution buffer. RNA was transferred onto a Hybond N^+ nylon membrane by capillary transfer. Northern hybridization analysis was performed by the procedure of Pawlowski *et al* (34). The 1.1-kb coding sequence of chitinase gene labelled with [α-^{32}P]dCTP, was used as probe.

Western blot analysis — Protein extraction and western blot analysis were performed as described by Chen *et al* (35). Young leaves (1 g) from uninfected T_1 plants were ground to a fine powder using liquid nitrogen and homogenized with the extraction buffer (35) supplemented with 10 mM β-mercaptoethanol. The extract was centrifuged at 18,000 x g for 20 min at 4 °C and the supernatant was used for western analysis. Protein concentration was estimated using Bradford's method (36). Twenty microgram aliquots of total protein were separated by SDS-PAGE in a 10% gel and transferred to a nitrocellulose membrane using a semi-dry transfer apparatus. Molecular weight markers (Rainbow marker) were from Amersham Pharmacia Biotech, Little Chalfont, England. The membrane was blocked using 3% gelatin and Tween-Tris buffered saline (TTBS) and probed with the chitinase antibody (a polyclonal rabbit antibody raised against barley chitinase) diluted to 1:1,000 (v/v). The second antibody, goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate from Bangalore Genei Pvt. Ltd., Bangalore, India was used at a dilution of 1:2,000. The membrane was treated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) colour reagent, until bands appeared.

Bioassay — *R. solani* was maintained on potato dextrose agar (37). Bioassay was done at the maximum tillering stage (45 days after transplanting to pots) as described by Lin *et al* (15). A small piece of agar block (5 mm thickness x 5 mm diameter) with fungal mycelium was placed inside the sheaths in each tiller and wrapped with wet cotton and parafilm. The inoculated plants (10 to 15) were maintained in a transgenic greenhouse. After seven days of inoculation, the sheath blight symptom was graded from 0 to 5 as described by Sriram *et al* (1). The virulence index was calculated using the following formula:

$$\text{Virulence index} = \frac{\text{Total grade points}}{\text{Maximum grade x No. of sheaths tested}} \times 100$$

Results

Stable transformation of PB1 — Twenty one-day-old scutellum-derived calli of PB1 were preincubated for four days and infected with *A. tumefaciens* LBA4404 (pSB1, pMKU-RF2). The binary vector, pMKU-RF2 (Fig. 1), is a derivative of pCAMBIA1301 that harbours rice chitinase gene (*chi*11) under maize *Ubi*1 promoter-intron. The plasmid pSB1 carries *vir*B, *vir*G and *vir*C of Bo542 Ti plasmid (24). The infected calli were co-cultivated for three days on Whatman No. 1 paper in the presence of 100 μM AS. After washes, the calli were placed on the selection medium (CI medium supplemented with 50 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime) for 14 days. Three cycles of selection, each for 21 days, were carried out. Of the 31 co-cultivated calli, 29 proliferated on hygromycin-containing medium (94%). Untransformed control calli did not proliferate beyond the third cycle. A portion from each hygromycin-resistant callus was stained for GUS activity. Seventeen out of 29 calli were GUS-positive, thus

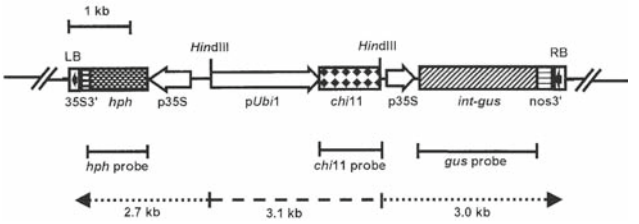

Fig. 1. Map of the T-DNA of pMKU-RF2. The T-DNA portion of the binary plasmid pMKU-RF2 harbours a p*Ubi*1-*chi*11 gene in the *Hin*dIII site of pCAMBIA1301. The T-DNA portion is delimited by right border (RB) and left border (LB). The *chi*11 has its own polyA signal (23). The regions covered by *gus*, *hph* and *chi*11 probes are marked. Right border junction fragments (>3.0 kb), left border junction fragments (>2.7 kb) and internal T-DNA fragment (3.1 kb) generated by *Hin*dIII digestion are indicated in dotted lines. p35S-Cauliflower mosaic virus 35S promoter; 35S3'-Cauliflower mosaic virus 3' region; *hph*-hygromycin phosphotransferase; p*Ubi*1-maize ubiquitin promoter-intron; *chi*11-rice chitinase gene; *int-gus*-β-glucuronidase gene with an intron.

resulting in 55% of stable transformation frequency. All GUS-positive calli were transferred to RM containing 40 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime. Though shoots regenerated from 17 calli, only 14 developed roots. All the 14 rooted plants were transferred to soil and grown in a transgenic greenhouse. Roots and leaves from all 14 plants were GUS-positive (data not shown).

Analysis of T_0 plants for T-DNA integration — T-DNA integration and copy number determination were done by performing Southern analysis. The map of the T-DNA region of pMKU-RF2 is shown in Fig. 1. The coding sequences of *gus* and *hph* were used as probes for right and left border analysis, respectively. In either case, *Hin*dIII will cleave at one of the two internal sites within the T-DNA and at another site from the plant DNA near the site of T-DNA integration. Thus, junction fragments with a portion of the T-DNA and a portion of plant DNA will hybridize to the respective probes.

DNA samples (10 μg) from control and thirteen T_0 transgenic plants were digested with *Hin*dIII and subjected to Southern analysis. Right border junction fragments that hybridize to 1.8-kb *gus* probe are expected to be longer than 3.0 kb which is the distance between the last *Hin*dIII site in the T-DNA and right T-DNA border (Fig. 1). In DNA analyzed from T_0 transgenic plants, junction fragments longer than 3.0 kb hybridized to the probe (Fig. 2A, B). No signal was detected in DNA from the control plant. One junction fragment was observed for plants 1, 12, 14 and 28, suggesting integration of a single copy of the T-DNA. One weak junction fragment signal was found for the plant 16. Multiple junction fragments of varying sizes were

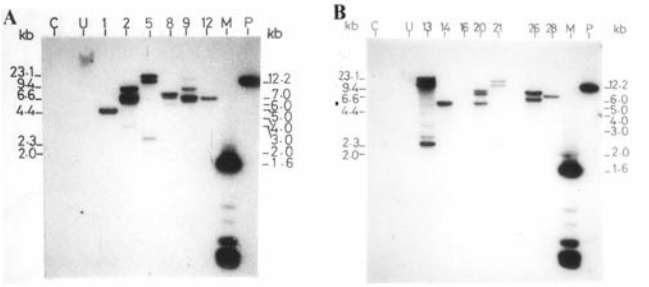

Fig. 2. Southern blot analysis of T_0 lines with *gus* probe flanking the right border. Transformation was done with LBA4404 (pSB1, pMKU-RF2). Panel A shows the results of analysis of T_0 lines 1, 2, 5, 8, 9 and 12. Panel B shows the results of T_0 lines 13, 14, 16, 20, 21, 26 and 28. Numbers on the top refer to T_0 lines. Plant DNA (10 μg) was digested with *Hin*dIII and analyzed. The blots were hybridized with [α-^{32}P]dCTP-labelled *gus* sequence. Lanes-C, DNA from untransformed (control) plant digested with *Hin*dIII; U, undigested DNA from T_0 plant 1; M, kb ladder (non-specific hybridization observed); P, 100 pg pMKU-RF2 digested with *Hin*dIII. Positions and sizes of λ-*Hin*dIII fragments (left) and kb ladder (right) are marked. The absence of strong signal in lanes containing undigested DNA from transgenic plant may be due to limited transfer during blotting from gels, used without depurination step.

detected for the remaining plants suggesting multicopy T-DNA insertions. In plants 5, 13 and 21, hybridization to fragments shorter than 3.0 kb is also seen. These may represent deletions/scrambling prior to T-DNA integrations. Analysis of plant 15, which was found to have multiple copies of the T-DNA, is not shown in Fig. 2B. The T_0 plant 8 showed one intense junction fragment and a second very weak junction fragment. Thus, T_0 plant 8 is inferred to have one complete T-DNA copy and a second truncated copy.

Junction sequence analysis was extended to the left border to validate copy number determination. DNA samples (10 μg) extracted from control and fourteen T_0 transgenic plants were digested with *Hin*dIII and the blots were probed with 1.1-kb *hph* coding sequence. The distance between the left T-DNA border and the first *Hin*dIII site in the T-DNA is 2.7 kb (*see* Fig. 1). Therefore, the junction fragments are expected to be longer than 2.7 kb. In plants 1, 8, 12, 14, 16 and 28, hybridization to single junction fragments was observed, whereas other plants showed multiple junction fragments (Fig. 3A, B). Thus, the presence of a single copy of the T-DNA in plants 1, 12, 14, 16 and 28 was confirmed with probes flanking both right and left borders. T_0 plant 16 showed a faint junction sequence with the right border (*gus*) probe, but it showed a clear junction fragment for left border probe (*hph*), suggesting that the right T-DNA portion may be truncated. In addition to the expected junction fragments longer than 2.7 kb,

shorter junction fragments (<2.7 kb) were seen in many T_0 plants with multiple T-DNA copies. These may represent truncated T-DNA transfer events. Hybridization patterns were similar in T_0 plants 2 and 15 and in plants 5, 13 and 21 with both probes, suggesting that they are siblings that regenerated from same transgenic calli. Hence, the total number of independent transformants was found to be eleven. Of these, five T_0 plants showed single copy T-DNA insertions at both right and left borders.

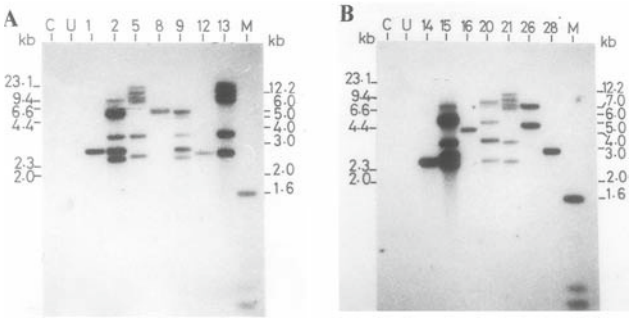

Fig. 3. Southern blot analysis of T_0 lines transformed with LBA4404 (pSB1, pMKU-RF2) using the *hph* probe flanking the left border. Panel A shows the results of analysis of T_0 lines 1, 2, 5, 8, 9, 12 and 13. Panel B shows the results of T_0 lines 14, 15, 16, 20, 21, 26 and 28. Numbers on the top refer to T_0 lines. Ten μg of DNA samples digested with *Hin*dIII were loaded in each lane and hybridized with [α-^{32}P]dCTP-labelled *hph* (1.1-kb) sequence. Lanes-C, DNA from untransformed (control) plant digested with *Hin*dIII; U, undigested plant DNA from T_0 line 1; M, kb ladder. Numerals on the sides indicate the sizes of λ-*Hin*dIII fragments (left) and kb ladder (right).

To confirm the presence of chitinase gene in transgenic plants, 1.1-kb *chi*11 coding sequence was used as probe. Digestion at two internal *Hin*dIII sites in the T-DNA will release a 3.1-kb fragment in the transgenic plants (Fig. 1) that will hybridize with the *chi*11 probe. The 3.1-kb internal T-DNA fragment was seen in all transgenic plants except in plants 16 and 28 (Fig. 4A, B). The control plant did not show a signal at 3.1 kb. Apart from the 3.1 kb fragment, many additional bands were detected in both control and transgenic plants. These bands represent signals from endogenous chitinase genes, which hybridized to the transgene (rice *chi*11), which we used as a probe.

Morphology of transgenic plants — All the transgenic plants grew normally till maturity. Seed setting was normal in all plants except the T_0 plant 28, which did not set seeds. T_0 plant 16 did not carry the *chi11* transgene. Hence, all further analyses were performed in T_0 plants 1, 12 and 14

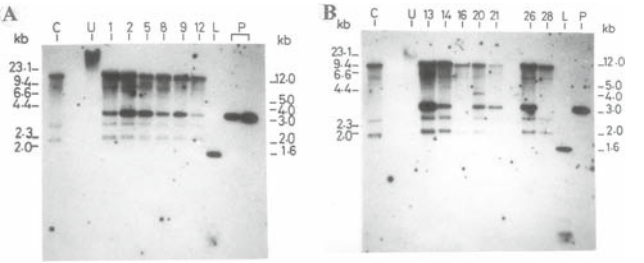

Fig. 4. Southern hybridization of T_0 lines with rice chitinase gene (*chi*11) present in the middle portion of the T-DNA. Panel A shows the results of analysis of T_0 lines 1, 2, 5, 8, 9 and 12. Panel B shows the results of T_0 lines 13, 14, 16, 20, 21, 26 and 28. Ten microgram of plant DNA was digested with *Hin*dIII and loaded in each lane. The blots were hybridized with [α-^{32}P]dCTP-labelled *chi*11 (1.1 kb) coding sequence. Lanes-C, DNA from untransformed (control) plant digested with *Hin*dIII; U, undigested DNA from T_0 plant 1; L, kb ladder. In Panel A, 'P' lanes have 100 pg and 500 pg of pMKU-RF2 digested with *Hin*dIII. 'P' lane in Panel B has 100 pg pMKU-RF2 digested with *Hin*dIII. The sizes of the standard DNA markers are positioned on the sides [λ-*Hin*dIII fragments (left) and kb ladder (right)].

that harboured single, complete copies of the T-DNA and in T_0 plant 8 with two copies of T-DNA.

Segregation pattern in T_1 plants — Segregation of the transgene in T_1 plants was studied to follow the inheritance of the transgene and to determine the transgene copy numbers (loci). Seeds obtained from selfed T_0 plants were germinated and scored for hygromycin-resistant growth. χ^2 test was performed to check the segregation pattern (Table 1). Mendelian segregation ratio of 3:1 for Hyg^R and Hyg^S was observed in the progenies of all the four T_0 lines. Both Southern analysis and segregation analysis suggest single copy transgene integration in T_0 plants 1, 12 and 14. Though Southern results of T_0 plant 8 showed one complete T-DNA and a second truncated T-DNA, segregation analysis showed that both T-DNA copies are at the same locus.

Expression of chitinase gene in T_1 plants — One representative T_1 plant from each of the T_0 lines 1, 12 and 14 was taken for comparing the chitinase transcript levels. Total RNA (10 μg) extracted from T_1 plants was subjected

Table 1. Segregation pattern of the *hph* gene in transgenic T_1 plants obtained from selfed T_0 lines

Selfed T_0	Number of seedlings			χ^2 value	P
plant line	Total	Hyg^R	Hyg^S	(3:1)	
8	30	21	9	0.4	>0.5
1	30	19	11	2.17	>0.1
12	30	20	10	1.11	>0.2
14	31	20	11	1.81	>0.2

to northern analysis using the rice chitinase gene as probe. A signal corresponding to 1.1-kb chitinase mRNA was seen in the lanes corresponding to the transgenic plants (Fig. 5). Hybridization was not seen in total RNA extracted from control plants suggesting that the endogenous rice chitinase expression is either very low or absent. These data suggest that the full length mRNA corresponding to the rice chitinase transgene is synthesized constitutively. T_1 plants from all three lines (1, 12 and 14) accumulated comparable levels of chitinase mRNA. T_1 plants from the T_0 line 8 also expressed chitinase gene constitutively (data not shown).

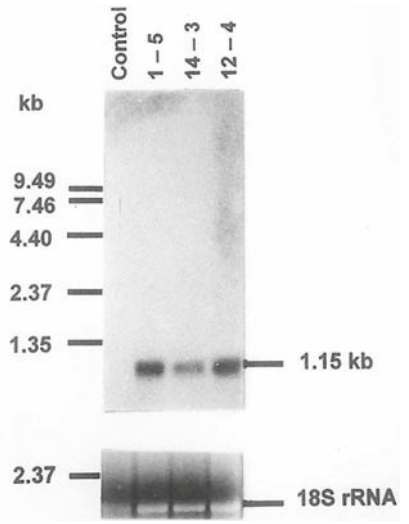

Fig. 5. Northern blot analysis of three representative T_1 plants of T_0 lines 1, 14 and 12. Ten microgram of total RNA was loaded per lane and fractionated in a 1.5% formaldehyde-agarose gel. Hybridization was performed using radiolabelled 1.1-kb chitinase gene as probe (top panel). A portion of ethidium bromide-stained gel before blotting is shown in the bottom panel to indicate the levels of 18S rRNA in all lanes. Positions and sizes of RNA size markers are marked on the left.

Western blot analysis was performed with two T_1 plants representing each of the three T_0 transgenic lines, 1, 12 and 14. Total soluble protein was extracted from the leaf tissues of control and transgenic plants. In each lane, 20 μg of total protein was loaded for SDS-PAGE analysis. Barley chitinase antibody (38) served as the primary antibody. The results (Fig. 6) showed high accumulation of a 35-kD chitinase in transgenic plants. Only a very weak signal was seen in control plant extracts. The size of the protein matched the expected relative molecular mass of 35,000 for chitinase (*chi*11) (23). In addition to the 35-kD protein, a band at 28 kD was also detected in all T_1 plants. The 28-kD protein may have been released by proteolysis. Comparable levels of chitinase protein accumulated in all the three transgenic lines. There was a slight difference in the levels of chitinase in two T_1 plants of the T_0 plant 1 presumably because one is hemizygous and the other is homozygous for the transgene. The T_1 plants of the T_0 line 8 also accumulated chitinase protein at levels comparable to those of lines 1, 12 and 14 (data not shown).

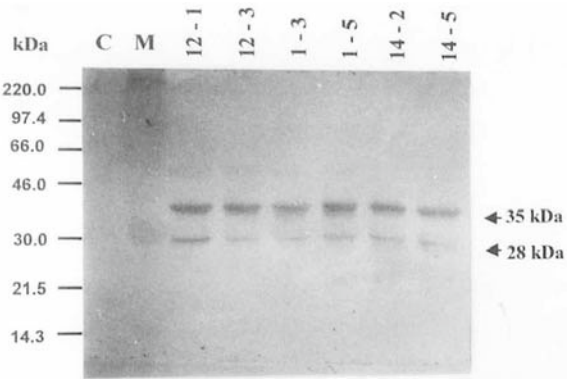

Fig. 6. Western blot analysis of two T_1 plants of each of the three T_0 lines 12, 1 and 14 for chitinase expression. Twenty microgram aliquots of total protein were loaded in each lane and separated by SDS-PAGE in 10% polyacrylamide gels. Rainbow marker was used as protein molecular weight standard (M). Protein extracted from leaves of non-transformed plant was loaded in the first lane (C). The numbers marked on top (e.g., 12-1) correspond to T_1 plants.

Bioassays with R. solani — Resistance of transgenic plants to sheath blight disease was evaluated by performing bioassays with *R. solani* (1). Approximately equal number of control and T_1 plants (ranging from 10 to 15) from each of the four T_0 transgenic lines (1, 8, 12 and 14) were subjected to bioassays with *R. solani*. Only Hyg^R T_1 plants (homozygous or hemizygous) were taken for bioassay. Bioassays were performed in a transgenic greenhouse at the maximum tillering stage (45 days after transplanting). Disease rating was scored after seven days of inoculation. The virulence index was determined as reported by Sriram *et al* (1) for the control and transgenic plants based on symptoms graded from 0 to 5. As shown in Table 2, the maximum level of resistance to *R. solani* was observed for the line 1, followed by the transgenic lines 12 and 8. The transgenic line 14 did not exhibit any resistance.

Analysis of T_2 plants — In order to obtain a homozygous transgenic line, seeds of T_1 plants of line 1 were germinated and scored for hygromycin resistance (Table 3). The T_1

Table 2. Bioassay of T_1 plants of T_0 lines 8, 12, 1 and 14 for resistance to *Rhizoctonia solani*

Transgenic line	Control plants		Transgenic plants	
	No. of plants	Average disease index[b]	No. of plants	Average disease index[b]
8	5	2.71	14	2.30
12[a]	12	3.26	15	2.64
1[a]	12	3.26	12	1.75
14	8	2.30	10	2.46

[a] Bioassay for transgenic lines 1 and 12 was done at the same time with one set of control plants.
[b] Scoring for disease index was done one week after infection.

plant 1-1 and the T_1 plant 1-3 are hemizygous for the transgene since their progeny segregated in a 3:1 ratio for Hyg^R and Hyg^S. All progeny plants of T_1 line 1-5 germinated on hygromycin confirming it to be a homozygous line. Thus, a homozygous Pusa Basmati-1 transgenic line has been obtained from a line that showed high level of resistance to *R. solani*.

Table 3. Identification of a homozygous line from selfed T_1 plants of T_0 line 1

Selfed T_1 plant line	Number of T_2 seedlings			χ^2 value	P
	Total	Hyg^R	Hyg^S		
1-1	34	22	12	1.92 (3:1)	>0.1
1-3	32	25	7	0.165 (3:1)	>0.5
1-5	34	34	0	0 (4:0)	>0.99

Plants 1-1, 1-3 and 1-5 were obtained from T_0 line 1.

Discussion

The effectiveness of constitutive expression of chitinase in conferring resistance to sheath blight disease in transgenic *indica* rice was initially evaluated by direct transformation. Lin *et al* (15) transformed the *indica* variety Chinsurah Boro II by PEG-mediated transformation using rice chitinase gene *chi*11 driven by CaMV 35S promoter. Resistance found in the transgenic plants showed a good correlation to the expression levels of chitinase. The *RC7* chitinase gene, induced in *R. solani*-infected rice plants, was recently introduced by Datta *et al* (17) into *indica* rice varieties, IR64, IR72 and Chinsurah Boro II by particle bombardment and PEG-mediated transformation. Homozygous transgenic lines showed sheath blight resistance up to 50% level. Gene silencing resulting from the integration of multiple copies of transgenes is a frequently encountered problem with direct transformation. Silencing of chitinase transgene in transgenic rice was reported by Chareonpornwattana *et al* (39).

Datta *et al* (16) performed *Agrobacterium*-mediated transformation to introduce CaMV 35S-*chi*11 into *indica* rice genotypes, Basmati 122, Tulsi and Vaidehi. The transgenic plants, confirmed by Southern and western analysis, showed improved resistance. Nishizawa *et al* (18) generated transgenic *japonica* rice plants by *Agrobacterium*-mediated transformation of class-I chitinase genes, *cht*-2 and *cht*-3. These transgenic plants showed enhanced resistance to the blast disease caused by *Magnaporthe grisea*. Both vacuolar (*cht*-2) and apoplastic (*cht*-3) chitinases were found to be equally effective in conferring blast resistance.

We obtained 11 independent transgenic rice plants with *Ubi*1-*chi*11 by *Agrobacterium*-mediated transformation. Of these, three plants (27%) harboured complete single copy T-DNA insertions. Interestingly, in many plants with multiple T-DNA copies (T_0 plants 2, 5, 9, 13, 15, 20 and 21) the number of junction fragments for the *hph* probe (left border region) was more than those for the *gus* probe (right border region). A similar observation was reported by Mohanty *et al* (40) in rice plants transformed with pTOK233. These results suggest that T-DNA scrambling and deletions could have occurred more frequently at the right border prior to T-DNA integration. Alternatively, formation of head to head, inverted T-DNA dimers around the right border would also explain the detection of lesser number of junction fragments around the right T-DNA border (41).

Southern analysis using the chitinase gene confirmed the presence of the transgene (*chi*11) in nine transgenic plants (Fig. 4A, B). The T_0 plant 16 had an intact LB region with the *hph* gene (Fig. 3) but lacked the *chi*11 transgene (Fig. 4). A faint signal observed with the *gus* probe may represent a second truncated T-DNA copy. The T_0 plant 28 is an interesting plant with one truncated T-DNA with RB-flanking region (Fig. 2) and a second truncated T-DNA with LB-flanking region (Fig. 3). Both T-DNA copies lacked *chi*11 (Fig. 4). The T_0 plant 28 could have been mistakenly considered as a single copy transgenic plant, if three probes had not been used for analysis.

A segregation ratio of 3:1 confirmed that T-DNA integrations in lines 1, 8, 12 and 14 are at single loci. Northern and western blot analysis showed high constitutive expression of chitinase in transgenic lines,

while its expression is very low and hardly detectable in control plants. Though chitinase expression was same in different transgenic lines, differences were observed in the levels of resistance to *R. solani*. The lack of good correlation between chitinase levels and the extent of disease resistance is surprising. However, a similar observation has been reported earlier in transgenic tobacco plants (14).

We report here a high frequency (55%, based on GUS^+ calli) of *Agrobacterium*-mediated transformation of an elite *indica* rice variety Pusa Basmati 1 with *chi*11 rice chitinase gene under maize *Ubi*1 promoter-intron. Three transgenic lines were found to have single-copy transgene integrations based upon detailed Southern analysis using probes from right border and left border regions and from the middle of the T-DNA. Progeny analysis at T_1 level confirmed single-locus T-DNA integrations in four transgenic lines. Bioassay in T_1 plants showed varying levels of resistance to *R. solani*. In the T_0 line 1 with a single transgene copy and a significant level of resistance to *R. solani*, a homozygous transgenic line has been established. Deployment of low copy number transgenic rice plants and the use of ubiquitin promoter-intron might be helpful in reducing the possibility of gene silencing that was observed in previous experiments with the same gene (39).

The results presented here highlight the importance of using three probes (LB-flanking, RB-flanking and middle portion with the transgene) in Southern analysis to ensure that the chosen transgenic plant has a single copy of the complete T-DNA.

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