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GENETIC TRANSFORMATION AND HYBRIDIZATION

Pyramiding transgenic resistance in elite indica rice cultivars against the sheath blight and bacterial blight

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Abstract Elite indica rice cultivars were cotransformed with genes expressing a rice chitinase (*chi11*) and a thaumatin-like protein (*tlp*) conferring resistance to fungal pathogens and a serine-threonine kinase (*Xa21*) conferring bacterial blight resistance, through particle bombardment, with a view to pyramiding sheath blight and bacterial blight resistance. Molecular analyses of putative transgenic lines by polymerase chain reaction, Southern Blot hybridization, and Western Blotting revealed stable integration and expression of the transgenes in a few independent transgenic lines. Progeny analyses showed the stable inheritance of transgenes to their progeny. Coexpression of chitinase and thaumatin-like protein in the progenies of a transgenic Pusa Basmati1 line revealed an enhanced resistance to the sheath blight pathogen, *Rhizoctonia solani*, as compared to that in the lines expressing the individual genes. A transgenic Pusa Basmati1 line pyramided with *chi11*, *tlp*, and *Xa21* showed an enhanced resistance to both sheath blight and bacterial blight.

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Keywords Bacterial blight · Chitinase · Disease resistance · Sheath blight · Thaumatin-like protein

Abbreviations BB: bacterial blight · FRI: functional resistance/susceptibility index · HRLH%: highest relative lesion height % · PB1: Pusa Basmati1 · ShB: sheath blight

Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops, from which nearly one third of world's population derive its principal source of calories. Rice yield has been affected by several abiotic and biotic factors, of the latter, the pests and diseases are important. Among various diseases affecting rice, sheath blight (ShB) caused by *Rhizoctonia solani* Kühn and bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Ishyama) Dye are highly pernicious. Estimated yield losses caused by sheath blight ranges from 8 to 50% (Savary et al. 2000), while the losses due to bacterial blight could reach up to 50%, when the pathogen infects the rice plants at maximum tillering stage (Adhikari et al. 1995). Owing to the inherent limitations of conventional breeding and environmentally hazardous nature of chemical pesticides, alternative strategies are being tried globally to enhance the resistance of crops against invading pathogens. Genetic engineering is a promising strategy to generate crop plants with disease resistance against economically important plant diseases like rice sheath blight and bacterial blight.

Conventionally, genetic engineering signifies genetic transformation of the crop with a single gene associated with the trait of interest (Ronald 1997; Zhang et al. 1998). Since disease resistance could rely on highly complex multigenic trait, single gene transformations, in general, result in

insufficient and/or narrow spectrum disease resistance (Neuhans et al. 1991; Anand et al. 2003) and there is always a possibility for reversal of resistance because of appearance of resistant strains of pathogens (Mew et al. 1992). Hence, genetic engineering of crop plants with (i) a combination of genes encoding/controlling interdependent or synergistic subcomponents of the disease-resistant trait to realize effective resistance against a particular disease or (ii) a combination of genes associated with different diseases to realize a wide-spectrum disease resistance would be more logical. An ingeniously planned genetic engineering involving a well-balanced expression of transgenes with different modes of action would ensure enhanced and durable resistance against pathogens. Availability of cloned disease resistance (Song et al. 1995) or defence genes (Huang et al. 1991; Velazhahan et al. 1998; Takakura et al. 2000) and enhanced transformation tools and strategies (Christou 1997; Chen et al. 1998; Kim et al. 2003) has made pyramiding of disease resistance genes in crop plants much easier. The capability of rice genome to integrate, coexpress, and inherit several transgenes, without undergoing developmentally and reproductively unfavorable phenotypic changes (Chen et al. 1998), highlights the potential of rice germplasm to be improved toward effective, stable, and inheritable disease resistance without compromising yield significantly. The success of multiple gene transformation or gene pyramiding is largely dependent upon the judicious selection of the genotypes of the crop to be transformed and the genes to be employed. The combination of genes encoding proteins involved in pathogen recognition and/or subsequent activation of signaling pathways leading to defence responses (Van Loon and Van Strien 1999) and those exhibiting direct antimicrobial properties (Broglie et al. 1991; Yun et al. 1998) by virtue of their degradative interaction with pathogen cell wall and cell membrane can be expected to improve the scope for generating crop plants with specific or wide-spectrum resistance against pathogens.

Conventional breeding (Yoshimura et al. 1995) and marker-assisted breeding (Huang et al. 1997; Singh et al. 2001) strategies achieved bacterial blight (BB) resistance genes-pyramided rice lines displaying wide-spectrum resistance against *Xoo* isolates/races (Kameswara Rao et al. 2002). Conventional crossing of two independent transgenic homozygous rice lines by Datta et al. (2002) pyramided *Xa21* gene, a chitinase gene, and a *Bt*-fusion gene into IR72 to confer multiple resistances against BB, ShB, and yellow stem borer. Combination of marker-assisted breeding (MAS) and genetic transformation yielded rice lines resistant to blast and BB by pyramiding *Pi1, Piz5* (major blast resistance genes), and *Xa21* (Narayanan et al. 2004). Genetic engineering of rice by Kim et al. (2003) to pyramid a maize ribosome-inactivating protein gene and a rice basic chitinase gene enhanced the ShB resistance of the transgenic rice lines. The pyramiding of *Xa21* and *gna* (*Galanthus nivalis* agglutinin) genes in rice by genetic transformation produced *Xa21* and *gna* coexpressing rice lines displaying BB and brown plant hopper resistance (Tang et al. 1999). Recently, Kalpana et al. (2006) showed coexpression of transgenic rice chitinase and thaumatin-like protein (TLP) in an elite indica rice line, which showed significantly higher level of ShB resistance than the chitinase or TLP transformants.

The present programme is concerned with pyramiding the disease resistance genes rice *chi11*, *tlp*, and *Xa21* in a few indica rice cultivars to confer effective, stable, and inheritable resistance against ShB and BB. *Xa21*, a major dominant gene for BB resistance, originally transferred from the wild rice *O. longistaminata* into the cultivar IR24 by Khush et al. (1990) and cloned by Ronald and coworkers (Ronald et al. 1992; Song et al. 1995), confers broad-spectrum resistance against diverse *Xoo* isolates/races in rice (Song et al. 1995; Wang et al. 1996; Zhang et al. 1998; Tu et al. 1998, 2000). Based on models from mammalian systems, *Xa21* has been suggested to play an important role in cell surface recognition of a pathogen ligand and subsequent activation of intracellular serine-threonine kinase(s) leading to a defense response (Song et al. 1995).

The other genes used in the study, the rice *chi11* and *tlp* encode, respectively, an enodochitinase and a TLP categorized as important pathogenesis-related (PR)-proteins (Van Loon and Van Strien 1999). PR-proteins accumulate in plant cells along with other defence-related molecules during plant's hypersensitive response (HR; Heath 2000) against pathogen attack. PR-proteins are classified on the basis of their amino acid sequences, serological relationships, and biochemical functions (Van Loon and Van Strien 1999) and several members of the PR-protein categories are well studied by various groups (Muthukrishnan et al. 2001). Different endochitinases (EC 3.2.1.14) belonging to PR-3, 4, 8, and 11 categories (Van Loon and Van Strien 1999) catalyze the hydrolysis of β-1,4 linkages between N-acetylglucosamine units of chitin, the major polysaccharide component of fungal cell wall (Broglie et al. 1991). A few chitinase cDNAs were isolated from rice (Huang et al. 1991; Takakura et al. 2000) and have been employed to transform rice cultivars to engineer ShB resistance (Lin et al. 1995; Datta et al. 2001; Kumar et al. 2003, Kalpana et al. 2006). TLP, belonging to PR-5 category, causes fungal cell membrane lysis and death by altering the cell membrane permeability (Yun et al. 1998). Several TLP genes were isolated from cereals including rice (Velazhahan et al. 1998). The transgenic rice plants overexpressing a rice *tlp* transgene have been reported to exhibit an enhanced resistance to ShB pathogen (Datta et al. 1999; Kalpana et al. 2006). A subgroup of TLPs has been shown to be β-1,3-glucan binding proteins (Osmond et al. 2001). Moreover, Grenier et al. (1999) showed the hydrolysis of β-1,3-glucans by some TLPs. The existing literature on the plant

antifungal PR-proteins implies that the fungal cell membrane-permeabilizing TLPs and the fungal cell wall hydrolyzing carbohydrases, such as chitinases and β-1,3-glucanases act in concert to kill fungal pathogens. Chitinases, TLPs, and β-1,3-glucanases are coregulated/coexpressed developmentally (Peumans et al. 2002) when the plants are subjected to abiotic stress (Hiilovaara-Teijo et al. 1999) and challenged by pathogens (Jacobs et al. 1999). Hejgaard et al. (1991) reported synergistic activity of barley TLP and chitinase against the fungi *Trichoderma viride* and *Candida albicans*. Recent attempt by Kalpana et al. (2006) to genetically engineer elite indica rice cultivars with rice chitinase and TLP suggested their synergistic expression in transgenic context and the resultant enhanced resistance to ShB.

In the present study, high yielding elite indica rice cultivars PB1, ASD16, ADT38, IR72, and White Ponni were cotransformed with *chi11*, *tlp*, and *Xa21* genes using immature embryos and mature seed-derived calli as explants. To ascertain the integration of transgenes in the host genome and their expression, molecular analyses of transgenic lines were conducted by polymerase chain reaction, Southern Blot hybridization, and Western Blotting analyses. Inheritability of coexpression of the transgenes was monitored till T_2 generation of the transgenic lines. The transgenic individuals were rigorously assayed for transgenic resistance against ShB and BB.

Materials and methods

Cotransformation of rice cultivars through particle bombardment

Transformation vectors used

Plant transformation vectors, (i) pMKU-RF2 (Kumar et al. 2003) harboring a 3.2 kbp chitinase gene expression cassette containing a 1.1 kbp rice *chi11* gene under the control of ubiquitin promoter and NOS polyA terminator, (ii) pGL2-*ubi-tlp* (Datta et al. 1999) harboring a 3.1 kbp TLP gene expression cassette containing a 1.1 kbp rice *tlp* gene under the control of a ubiquitin promoter and NOS terminator, and (iii) pC822 (Song et al. 1995) harboring a 9.6 kbp rice genomic DNA insert containing the *Xa21* gene expressing a receptor kinase-like protein conferring bacterial blight resistance were used in the cotransformation experiments. A 10 μg equimolar mixture of the three transformation vectors was used for coating 5 mg of gold particles (Bio-Rad Laboratories, USA), as per the manufacturer's instructions.

Particle bombardment of immature embryos

Immature seeds of the elite indica rice cultivars ASD16, ADT38, IR72, IR64, and White Ponni, harvested 12–14 days postanthesis from Paddy Breeding Station (PBS), Tamil Nadu Agricultural University (TNAU), Coimbatore, India, were manually dehusked and surface-sterilized with 0.1% mercuric chloride for 5 min. The immature embryos isolated aseptically under a stereomicroscope (Leica, Switzerland) were plated with their scutella up (Datta et al. 2001) onto CC medium (Potrykus et al. 1979) containing 2.0 mg l^{-1} 2,4-D and cultured for 2 days in the dark at $25 \pm 2^{\circ}$C. After 2 days, precultured embryos were bombarded twice using PDS-1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, Hercules, USA) at 1100 psi and 27 in. of Hg vacuum, according to Zhang et al. (1998). The putative transformants were selected and regenerated on a CC-based medium containing 50 mg l^{-1} hygromycin B (Boehringer Mannheim GmbH, Germany).

Particle bombardment of mature seed-derived calli

Manually dehusked seeds of PB1 (obtained from PBS, TNAU) were surface sterilized with 70% ethanol for 3 min followed by 0.1% mercuric chloride for 5 min. After washing with sterile water, the seeds were plated onto MS (Murashige and Skoog 1962) medium containing 2.5 mg l^{-1} 2,4-D and incubated in the dark at $25 \pm 2^{\circ}$C for 3 weeks. Embryogenic calli developing from the seeds were subcultured onto fresh medium and allowed to proliferate for another 2 weeks. Hard friable calli were selected and cut into small pieces of 2–3 mm size, 3–4 days prior to bombardment. Calli were bombarded twice following standard protocols (Zhang et al. 1998). After three rounds of selection on hygromycin B (50 mg l^{-1}), plants were regenerated on MS medium supplemented with 3.0 mg l^{-1} BAP (BA; N^6-benzyladenine; Sigma, USA) and 1.5 mg l^{-1} NAA (α-naphthalene acetic acid).

Molecular and biochemical analyses of putative (T_0) transgenic lines

Polymerase chain reaction

Genomic DNA was isolated from putative transgenic plants and nontransgenic control plants, following Dellaporta et al. (1983) and PCR was performed to amplify *Xa21* gene. Forward primer U1 corresponding to the 3′ untranslated region of the *Xa21* gene (5′-CGATCGGTATAACAGCAAAAC-3′) and reverse primer I1 (5′-ATAGCAACTGATTGCTTGG-3′) corresponding to the center of the intron (9.6 kb *Kpn*I fragment of *Xa21* gene has a single large ORF of 3075 bp intervened by a 843 bp intron; Song et al. 1995) were used to amplify a 1.4 kbp-long internal fragment of *Xa21*, as described by Wang et al. (1996). Amplification was performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., USA).

Southern Blot hybridization analysis

Ten micrograms of genomic DNA extracted from putative transgenic plants and nontransgenic control plants and 5 ng of plasmid DNAs with the transgenes of the study (positive control) were digested overnight with appropriate restriction endonucleases (Bangalore Genei Pvt. Ltd., India). The products were electrophoresed on 1.0% (w/v) agarose gels and then transferred onto nylon membranes (Hybond$^+$, Boehringer and Mannheim, UK). The blots were subsequently hybridized overnight with radiolabeled probes prepared by random primer oligo labeling method (Random Primer Labeling Kit, Bangalore Genei Pvt. Ltd., India). Hybridization and autoradiography were carried out following standard procedures (Sambrook et al. 1989)

To demonstrate the transgenic integration of *chi11*, the genomic DNAs were digested with *Hin*dIII to release the 3.2 kbp chitinase expression cassette and blotted onto the membrane. The blot was hybridized with α-^{32}P dCTP-labeled 1.08 kbp *chi11* coding sequence obtained by digesting pMKU-RF2 with *Pst*I. To confirm the transgenic integration of *tlp* gene, the genomic DNAs were digested with *Hin*dIII to release the 3.1 kbp TLP expression cassette and hybridized with α-^{32}P dCTP-labeled 1.1 kbp TLP coding sequence obtained by digesting 3.1 kbp TLP expression cassette with *Bam*HI. Similarly, to confirm the transgenic integration of *Xa21*, the genomic DNAs were digested with *Eco*RV to release a 3.8 kbp (containing most of the coding sequence of the *Xa21*; Wang et al. 1996) internal fragment of *Xa21* (9.6 kbp) and hybridized with α-^{32}P dCTP-labeled 3.8 kbp *Xa21* coding sequence obtained by digesting pC822 with *Eco*RV.

Western Blotting analysis

Leaves (250 mg) from putative transgenic plants and nontransgenic control plants were ground with 150 μl of ice-cold phosphate-buffered saline. The extracts were centrifuged at 12000 rpm for 10 min at 4°C. The supernatants were collected and the proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel (12%) electrophoresis (Mini-PROTEAN®II cell, Bio-Rad Laboratories, USA), following standard procedure (Laemmli 1970).

Western Blotting analysis was carried out following the method described by Gallagher et al. (1995). Fractionated proteins were transferred onto a nitrocellulose membrane (Protran BAS 5 Cellulosenitrat, Schleicher and Schuell, Germany) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, USA). The membranes were then probed with either *anti*barley chitinase antibody (gifted by Dr. S. Muthukrishnan, Kansas State University, USA) or *anti*tobacco TLP antibody (gifted by Dr. Legrand, Strasbourg, Cedex, France). The polypeptides recognized by the specific antibodies were detected using a goat *anti*rabbit IgG-alkaline phosphatase conjugate by incubating the membrane in the dark with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma-Aldrich Co., USA).

Cotransformation efficiency

Cotransformation efficiency refers to the ratio of number of putative transgenic lines harboring/expressing more than one gene of interest (at DNA level; as estimated by PCR or Southern analysis or at protein level; as estimated by Western analysis) to the total number of explants bombarded.

Progeny analysis

Of the three T_0 PB1 lines expressing all the three transgenes (*chi11*, *tlp*, and *Xa21*), a morphologically normal and fertile line SM-PB1-1 was forwarded to generate T_1 progeny. The T_0 lines forwarded for T_1 generation also included PB1 lines expressing either *chi11* or *tlp* and an ASD16 line expressing *Xa21*. Nurseries were raised with the seeds collected from five T_0 PB1 lines viz., SM-PB1-1 (expressing *chi11*, *tlp*, and *Xa21*), SM-PB1-5 (expressing *tlp*), SM-PB1-9, 12, and 13 (expressing *chi11*), and an ASD16 line, SM-ASD16-2 (expressing *Xa21*). The resulting T_1 progenies of these transgenic lines were analyzed by PCR and/or Western Blotting analyses. Plants of T_1 progeny showing stable integration and expression of the transgenes were forwarded to generate T_2 progeny and the plants were analyzed by PCR and/or Western Blotting analyses.

Evaluation of ShB and BB resistance in T_1 and T_2 progeny

Bioassay for ShB resistance

The PB1 transgenic lines SM-PB1-1 (expressing *chi11*, *tlp*, and *Xa21*), SM-PB1-5 (expressing *tlp*), and SM-PB1-9 (expressing *chi11*) were evaluated for ShB resistance along with suitable nontransgenic control plants. Two different ShB assay methods viz., "bioassay using detached leaves" and "bioassay using leaf sheaths intact" were employed for screening the transgenic lines, as described by Kumar et al. (2003). All the inoculations were carried out with 5 mm mycelial discs obtained from 3-day-old *Rs*7 (a virulent isolate of *R. solani*; Krishnamurthy et al. 1999) maintained on potato dextrose agar (250.0 g l^{-1} potato, 20.0 g l^{-1} dextrose, 20.0 g l^{-1} agar, pH 7.0).

In bioassay using detached leaves, observations were made at 24 h intervals and infection cushions were counted under a stereomicroscope, 72 h after inoculation (HAI). In bioassay using leaf sheaths intact, observations were made up to 7 days at 24 h intervals and functional re-

sistance/susceptibility index (FRI) was calculated. At 168 HAI, the highest relative lesion height percentage (HRLH%; http//www.riceweb.org/ses/cd_diseases_d.htm) and the total lesion spread were estimated. HRLH% was calculated using the formula,

$$\text{HRLH\%} = \frac{\text{Length of the highest lesion (cm)}}{\text{Plant height (cm)}} \times 100$$

Bioassay for BB resistance

Preparation of bacterial suspension

A virulent isolate of *Xoo* (Tamil Nadu Rice Research Institute, Aduthurai, India) maintained on PSA (5.0 g l^{-1} peptone, 20.0 g l^{-1} sucrose, 0.5 g l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g l^{-1} $FeSO_4 \cdot 7H_2O$, 2.0 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$, 20.0 g l^{-1} agar) was used as source of inoculum. A loopful of bacteria was streaked onto a PSA slant and allowed to grow for 48 h at 28°C. Bacterial suspension was prepared by adding sterile distilled water (10 ml) into the 48 h-old culture and adjusting bacterial population to 10^9 cells/ml. All inoculations were made within 30–60 min after preparing the bacterial suspension.

Inoculation by leaf clipping

The PB1 line SM-PB1-1 (expressing *chi11*, *tlp*, and *Xa21*) and the ASD16 line, SM-ASD16-2 (expressing *Xa21*) maintained in a greenhouse for transgenic plants were evaluated for BB resistance. Leaf clipping method (Kauffman et al. 1973) was employed for inoculating 60-to-80-day-old plants (maximum tillering stage). After inoculation, the plants were covered with polyethylene bags with inner surface sprinkled with sterile distilled water to maintain plants under higher relative humidity for better symptom development. From each line, five plants (5–10 leaves/plant) were inoculated along with suitable nontransgenic control plants. Lesion length was measured 14 days after inoculation and based on the length of the developed lesions, the plants were categorized as resistant (1–3 cm), moderately resistant (3–6 cm), and susceptible (> 9 cm), as described by Mew and Vera Cruz (1979).

Statistical analyses

All the bioassay experiments were carried out in completely randomized design (CRD) with adequate replications. Duncan's multiple range test (DMRT; Gomez and Gomez 1984) was used to compare treatment means using the software IRRISTAT Version 3.1 (Biometrices Unit, International Rice Research Institute, Manila, The Philippines).

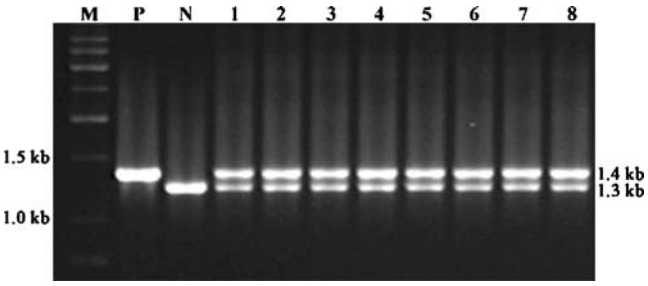

Fig. 1 PCR amplification of *Xa21* gene in the putative transgenic (T_0) indica rice lines. *Lane* M, 1.0 kb marker; P, positive control (pC822); N, negative (untransformed) control; 1–8, putative transgenic lines, SM-PB1-1, 2, 3, 7, SM-ASD16-2, SM-ADT38-1, SM-WP-1 and 2, respectively

Results and discussion

One of the major challenges in agriculture worldwide is to control the great yield loss caused by pests and pathogens. Realization of this objective, however, in an environmental-friendly way necessitates integrated efforts from plant breeders, pathologists, and genetic engineers. Despite successful genetic engineering of crop plants for the expression of transgene mediated disease resistance, the exhaustive efforts ended up with crops exhibiting insufficient or narrow-spectrum disease resistance or reversal of disease resistance. As a sequel, stacking or pyramiding of genes emerged as an alternative to engineering monogenic resistance with a sole aim of enhancing the durability of resistance against individual members of the pathodeme (Singh et al. 2001; Datta et al. 2002). In the present study, with the view to conferring higher level resistance against sheath blight along with resistance to bacterial blight, a few elite indica rice cultivars have been transformed with rice *chi11*, *tlp*, and *Xa21* genes. The transformants were molecularly characterized for the stable and inheritable coexpression of the transgenes and rigorously assayed for the associated resistance against ShB and BB.

Integration of *Xa21* transgene in the putative T_0 transgenic lines was ascertained by PCR as well as Southern Blot hybridization analysis. PCR of DNA from transgenic plants with *Xa21* kinase domain- and intron-specific primers revealed amplification of the expected 1.4 kbp fragment from four lines of the cultivar PB1 (SM-PB1-1, 2, 3, and 7), one line of the cultivar ASD16 (SM-ASD16-2), one line of the cultivar ADT38 (SM-ADT38-1), and two lines of the cultivar White Ponni (SM-WP-1 and 2) (Fig. 1). Apart from the transgene-specific 1.4 kbp band, a common band of 1.3 kbp was observed in both transgenic and nontransgenic plants. Similar banding pattern was reported earlier from PCRs of *Xa21* transgenic lines performed with the same set of primers (Wang et al. 1996; Tu et al. 1998) and the product was attributed to a cross-amplification of a member of the *Xa21* multigene family. Southern Blot hybridization analyses of *Eco*RV-digested DNA of transgenic lines with 3.8 kbp *Xa21*

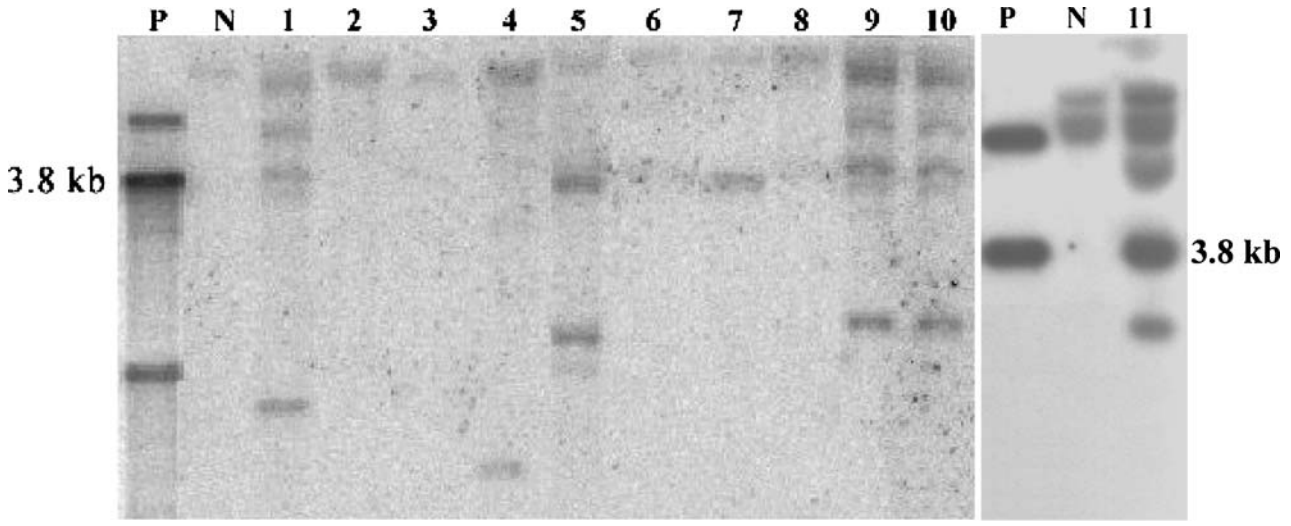

Fig. 2 Southern Blot hybridization analysis of *Xa21* gene in the putative transgenic (T_0) lines. *Lane* P, positive control (pC822-*Eco*RV digest); N, negative control; 1–11, putative transgenic lines, SM-PB1-1, 4, 5, 6, 7, 8, SM-ADT38-1, SM-IR72-1, SM-WP-1, 2 and SM-ASD16-2, respectively

coding sequence probe (released from the transformation vector pC822 by *Eco*RV digestion) revealed a 3.8 kbp hybridization signal from the transgenic lines SM-PB1-1 and 7, SM-ADT38-1, SM-WP-1 and 2, SM-ASD16-2, while such signal was absent from nontransgenic control plants. In addition, nonendogenous transgene-specific bands of different sizes were also observed in some of the transgenic lines (Fig. 2). These transgene-specific bands may be due to transgene rearrangement during integration (Tang et al. 1999; Datta et al. 2002). Moreover, a high molecular weight (> 9.0 kbp) band was observed in both the transgenic and nontransgenic lines. This signal may be corresponding to the one reported earlier to represent the polymorphic family members of the *Xa21* gene family (Wang et al. 1996; Tu et al. 1998, 2000; Tang et al. 1999).

Southern Blot hybridization analysis of *Hin*dIII-digested genomic DNA of transgenic lines with 1.08 kbp *chi11* coding sequence detected a 3.2 kbp hybridization signal representing the *Hin*dIII-released *chi11* gene expression cassette from four transgenic lines (SM-PB1-1, 9, 12, and 13) of the cultivar PB1 and a transgenic line of the cultivar ADT38 (SM-ADT38-1) revealing a stable integration of *chi11* transgene expression cassette in their genomes. In addition to this, two low molecular weight bands (< 3.2 kbp) were detected in both transgenic and nontransgenic lines (Fig. 3). Lin et al. (1995) used *chi11* coding sequence as probe to hybridize the *Hin*dIII-digested genomic DNA of transgenic and nontransgenic plants and observed two additional hybridization signals in both transgenic and nontransgenic lines, apart from the expected band in transgenic plants.

Southern Blot hybridization analysis of *Hin*dIII-digested DNA of transgenic plants with 1.1 kbp *tlp* coding sequence identified a hybridization signal of 3.1 kbp representing the

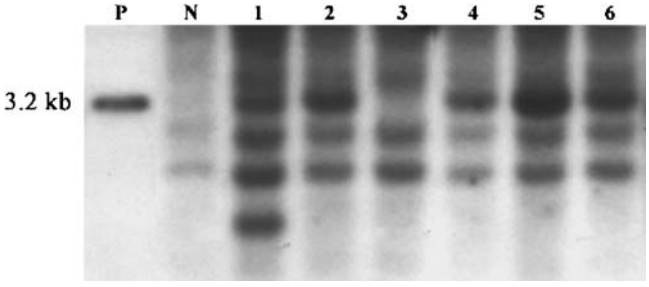

Fig. 3 Southern Blot hybridization analysis of *chi11* gene in the putative transgenic (T_0) lines. *Lane* P, positive control (pMKU-RF2-*Hin*dIII digest); N, negative control; 1–6, putative transgenic lines, SM-PB1-1, 9, 10, 12, 13 and SM-ADT38-1, respectively

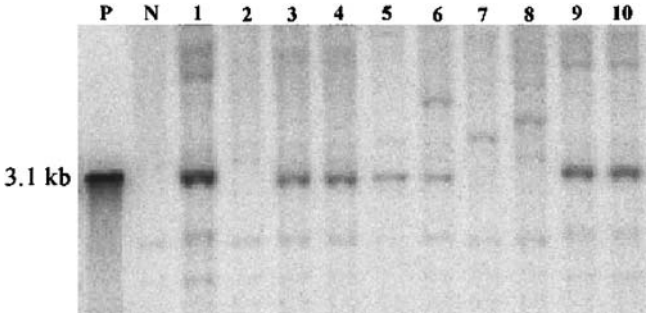

Fig. 4 Southern Blot hybridization analysis of *tlp* gene in the putative transgenic (T_0) lines. *Lane* P, positive control (pGL2-*Hin*dIII digest); N, negative control; 1–10, putative transgenic lines, SM-PB1-1, 4, 5, 6, 7, 8, SM-ADT38-1, SM-IR72-1, SM-WP-1 and 2, respectively

3.1 kbp *tlp* expression cassette released by *Hin*dIII digestion from five independent transgenic lines of the cultivar PB1 (SM-PB1-1, 5, 6, 7, and 8) and two lines of the cultivar White Ponni (SM-WP-1 and 2). Apart from this, a common band was found to be associated with the putative transgenic and nontransgenic plants (Fig. 4).

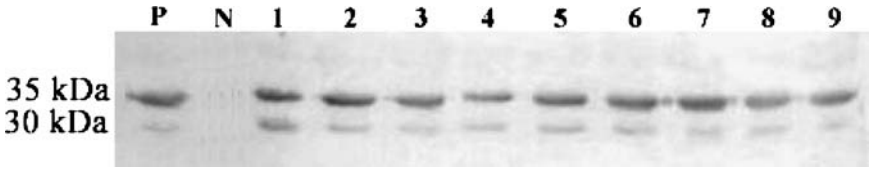

Fig. 5 Western Blotting analysis of chitinase expression in the putative transgenic (T_0) lines. *Lane* P, positive control (rice chitinase); N, negative control; 1–9, putative transgenic lines, SM-PB1-1, 2, 3, 9, 12, 13, SM-ADT38-1, 2 and SM-ASD16-3, respectively

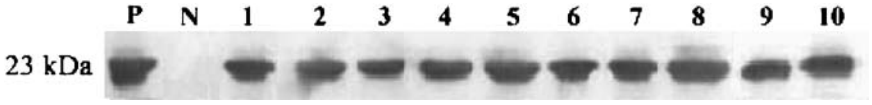

Fig. 6 Western Blotting analysis of TLP expression in the putative transgenic (T_0) lines. *Lane* P, positive control (pearl millet TLP); N, negative control; 1–10, putative transgenic lines, SM-PB1-1, 2, 3, 5, 6, 7, 8, SM-ASD-16-1, SM-WP-1 and 2, respectively

The expression of *chi11* and *tlp* transgenes in transgenic T_0 lines were studied by Western Blotting analyses of their total soluble leaf protein extracts against *anti*barley chitinase antiserum and *anti*tobacco TLP antiserum, respectively. The *anti*barley chitinase antiserum detected the expression of a 35 kDa polypeptide in six lines of the cultivar PB1 (SM-PB1-1, 2, 3, 9, 12, and 13), two lines of the cultivar ADT38 (SM-ADT38-1 and 2), and one line of the cultivar ASD16 (SM-ASD16-3). Apart from the expected 35 kDa signal corresponding to chitinase polypeptide, a 30 kDa signal was also found to be associated with all the transgenic lines (Fig. 5). This 30 kDa polypeptide may be a truncated version of the original 35 kDa protein released by proteolytic processing of the latter (Lin et al. 1995). Both these signals were not detected from nontransgenic control plants. Similarly, *anti*tobacco TLP antibody was able to detect the expression of a 23 kDa polypeptide in seven T_0 lines of the cultivar PB1 (SM-PB1-1, 2, 3, 5, 6, 7, and 8), one line of the cultivar ASD16 (SM-ASD16-1), and two lines of the cultivar White Ponni (SM-WP-1 and 2) (Fig. 6). Earlier, the expression of TLP (23 kDa) in transgenic lines of the indica rice cultivars Chinsurah Boro II, IR72, and IR501500 was demonstrated by Datta et al. (1999).

Results of the molecular analyses (PCR, Southern Blot hybridization analysis and Western Blotting analysis) of the T_0 lines showed the integration and expression of all the three genes (*chi11*, *tlp*, and *Xa21*) in at least three lines of the cultivar PB1 (SM-PB1-1, 2, and 3), *tlp* and *Xa21* in one line of the cultivar PB1 (SM-PB1-7) and two lines of White Ponni (SM-WP-1 and 2) and *chi11* and *Xa21* in one line of the cultivar ADT38 (SM-ADT38-1) (Fig. 1–6).

In our experiments, cotransformation efficiency (with respect to the genes of interest) of 1.7, 0.20, and 0.54% was observed in PB1, ADT38, and White Ponni, respectively (Table 1). Previously, a cointegration frequency of $>70\%$ was reported in japonica cultivars (Chen et al. 1998; Tang et al. 1999). However, low frequency of transformation is common among indica cultivars. Christou et al. (1991) reported a transformation efficiency of 0.80 and 3.20% in IR72 and IR36, respectively, using immature embryos as explants. The low frequency of transformation may be attributed to the recalcitrance posed by indica cultivars in getting transformed (Sivamani et al. 1996) or to the transgene rearrangement during integration (Tang et al. 1999; Datta et al. 2002).

The progeny (T_1) analysis of SM-PB1-1, a transgenic line of the cultivar PB1 that showed stable expression of all the three (*chi11*, *tlp*, and *Xa21*) genes was carried out along with four other PB1 (SM-PB1-5, 9, 12, and 13) lines expressing either *chi11* or *tlp* and one line of the cultivar ASD16 (SM-ASD16-2) expressing *Xa21*. The analysis showed that the transgenes were inherited as a single Mendelian trait (3:1 ratio) in all the lines (data not shown), except the line SM-PB1-1 carrying *chi11*, *tlp*, and *Xa21* genes. A 3:1 segregation ratio was observed for *chi11* and *tlp,* as against a 1:1 ratio for *Xa21* (Table 2). Tang et al. (1999) observed such a distorted segregation pattern in particle bombardment-mediated co-transformation experiments involving four transgenes (*gna*, *Xa21*, *gusA*, and *hpt*) and correlated it with two independent integration events (integration at two unlinked loci) involving all the four genes. Gahakwa et al. (2000) encountered such an unusual segregation pattern in 15% of the transgenic rice lines analysed. Several other groups have also reported such a distorted segregation ratio in transgenic rice lines (Peng et al. 1995; Fu et al. 2000). Progeny analysis by PCR/Western Blotting of T_2 generation of the earlier-described lines showed stable inheritance and expression of transgenes. Though segregation of transgenes in the progeny was evident (Fig. 7; Table 2), no significant variation in the levels of chitinase and TLP expression between T_1 and T_2 progenies was noticed (data not shown). The previous attempt by Kalpana et al. (2006) to coexpress *chi11* and *tlp* in the indica rice line ended up in silencing of chitinase transgene in the T_1 generation.

ShB and BB resistance of the transgenic lines were assessed by bioassays performed with individuals of T_1 and T_2 generations. For the bioassay, the individuals were selected

Table 1 Molecular analyses of putative transformants (T_0) of elite indica rice cultivars

Variety	No. of explants bombarded	No. of lines regenerated	No. of lines positive for the gene(s) of interest						Cotransformation efficiency (%)
			chi11[a]	*tlp*[a]	*Xa21*[b]	*chi11* + *Xa21*	*tlp* + *Xa21*	*chi11* + *tlp* + *Xa21*	
PB1	235 (MSC[c])	17	3	3	–	–	1	3	1.7
ASD16	585 (IE[d])	9	1	1	1	–	–	–	–
ADT38	490 (IE)	6	–	–	–	1	–	–	0.20
White Ponni	365 (IE)	10	–	–	–	–	2	–	0.54

[a]Plants were analyzed by Western and/or Southern analysis.

[b]Plants were analyzed by PCR and/or Southern analysis.

[c]Mature seed-derived calli.

[d]Immature embryos.

Table 2 Progeny (T_1 and T_2) analysis of the pyramided transgenic PB1 line

Generation studied	Transgenic line	No. of plants analyzed	No. of plants positive for the gene(s) of interest					Segregation ratio	χ^2
			chi11[a]	*tlp*[a]	*Xa21*[b]	*chi11* + *tlp*	*chi11* + *tlp* + *Xa21*		
T_1	SM-PB1-1 (*chi11* + *tlp* + *Xa21*)	23	–	1	–	4	13	3:1 (*chi11*)	0.003
								3:1 (*tlp*)	0.032
								1:1 (*Xa21*)	0.20
T_2	SM-PB1-1 (*chi11* + *tlp* + *Xa21*)	21	–	–	–	8	9	3:1 (*chi11*)	0.099
								3:1 (*tlp*)	0.099
								1:1 (*Xa21*)	0.21

[a]Plants were analyzed for the inheritance and expression of transgene(s) through Western.

[b]Plants were analyzed for the inheritance and expression of transgene through PCR.

only on the basis of the level of expression of transgenic chitinase and TLP (as assessed by Western Blotting), since the previous results did not evidence any correlation between homozygosity and disease resistance, though the transgene expression level was found to influence the resistance (Kim et al. 2003).

In the present study, bioassay for ShB resistance using detached leaves showed the appearance of *Rs*7 infection cushions away from the site of inoculation of *Rs*7, probably because of the pathogen's development of new hyphae, 48 HAI. However, the number of infection cushions formed was significantly lesser in the leaves of PB1 lines expressing either *chi11* or *tlp*, as compared to their respective non-transgenic controls. In the PB1 (SM-PB1-1) line expressing both *chi11* and *tlp*, the number of infection cushions was even lesser than that in the lines expressing either *chi11* or *tlp* (Table 3). Apart from the reduction in the number of infection cushions, the leaves of transgenic plants also exhibited reduction in the size of the lesions with characteristic browning around the lesions, signifying effective restriction of pathogenic invasion (Fig. 8A–C). In the leaves of nontransgenic controls, light grayish lesions (1.5–2.0 cm) with thin brown borders were produced. The gradual enlargement of these grayish lesions caused morbid yellowing and subsequent drying of the infected leaf, 72 HAI (Fig. 8D).

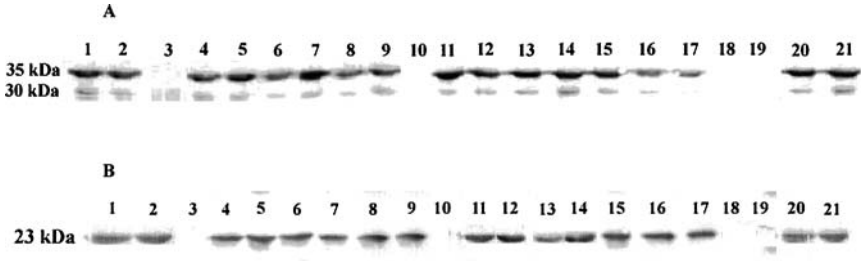

Fig. 7 Western Blotting analysis for the coexpression of chitinase and TLP. Coexpression of chitinase **A** and TLP **B** in the T_2 progenies (*Lanes* 1–21) of the pyramided line SM-PB1-1 harboring *chi11*, *tlp*, and *Xa21*

Table 3 Assessment of sheath blight resistance in transgenic lines by bioassay using detached leaves

Line	No. of infection cushions[a]	
	T_1	T_2
SM-PB1-9 (*chi11*)	7.27 c	7.93 b
SM-PB1-5 (*tlp*)	6.47 b	7.52 b
SM-PB1-1 (*chi11* + *tlp* + *Xa21*)	1.47 a	2.02 a
Untransformed PB1	35.93 d	40.21 c

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

[a]Replicated 15 times

In the bioassay on intact leaf sheaths, the transgenic PB1 lines did not show any disease lesion till 48 HAI, whereas in the nontransgenic PB1 plants, blanch lesions with a thin brown border were formed within this time period. These lesions slowly turned grayish and enlarged in size covering the entire leaf sheath within 168 HAI (Fig. 9D). Contrastingly, in all the transgenic lines (SM-PB1-9, 5, and 1) expressing *chi11* and/or *tlp*, much smaller-sized oblong grayish lesions surrounded by thick brown borders appeared (Fig. 9A–C). A precocious browning at the site of inoculation was observed only in the transgenic plants and such a browning might play a major role in arresting the pathogen spread. This was obvious from the drying of infected leaf sheaths in untransformed control plants, while leaf sheaths of transgenic plants expressing *chi11* and/or *tlp* remained green despite ShB infection (data not shown). This clearly demonstrated that the pathogen spread was very much restricted in transgenic lines possibly due to an extensive browning at the site of inoculation, a defense response very similar to hypersensitive reaction. Groth and Nowick (1992) suggested that the resistance to the spread of *R. solani* in rice could be consequent to production of oxidised phenolics (dark zone around lesions). Yamamato et al. (2000) observed an extensive browning and necrotic symptoms on transgenic grapevine plants expressing a rice chitinase (*RCC2*) gene leading to a hypersensitive reaction against powdery mildew pathogen *Uncinula necator*. The rapid cell death consequent to pathogen invasion was considered to be a common expression of resistance/defence in plants against the invading pathogen (Heath 2000).

Evaluation of ShB resistance using FRI revealed that there was a significant rise in FRI of transgenic PB1 (SM-PB1-9, 5, and 1) lines over nontransformed ones at 72, 96, 120, 144, and 168 HAI. Such an increase in FRI over control was maximum (344% in SM-PB1-9, 267% in SM-PB1-5, and 482% in SM-PB1-1) at 120 HAI. Similar increase in FRI of PB1 line SM-PB1-1 expressing both *chi11* and *tlp* was 253%, as compared to 168% and 159% in SM-PB1-9 (*chi11*) and SM-PB1-5 (*tlp*) respectively, at 168 HAI (Fig. 10). Kumar et al. (2003) observed a gradual increase in FRI values in untransformed lines, whereas a gradual initial increase followed by steep rise in FRI values in transgenic rice lines expressing *chi11* was reported.

Similarly, transgenic PB1 plants expressing *chi11* and/or *tlp* recorded a lower HRLH% while restricting lesion spread, when compared to untransformed PB1 (Table 4). Total lesion spread on leaf sheath of the transgenic line SM-PB1-1 (expressing *chi11* and *tlp*), 168 HAI was 4.90 cm as compared to 7.94 and 8.04 cm in SM-PB1-9 (expressing *chi11*) and SM-PB1-5 (expressing *tlp*) respectively, while total lesion spread on nontransgenic PB1 was 15.5 cm. Reduction in HRLH% and total lesion spread was more significant in the transgenic PB1 line (SM-PB1-1) expressing both *chi11* and *tlp* than the lines expressing either *chi11* or *tlp* (Table 4).

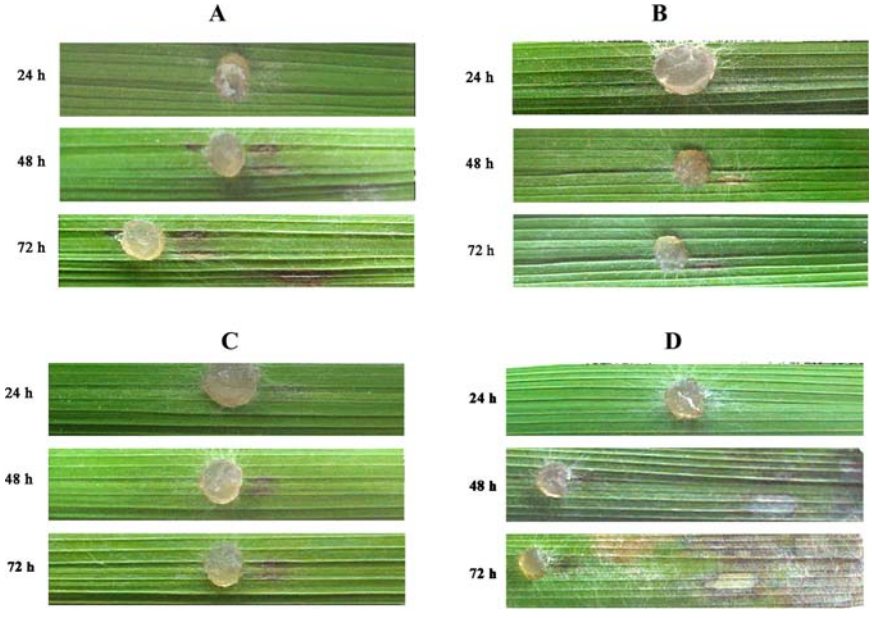

Fig. 8 Bioassay using detached leaves in the transgenic (T_1) PB1 lines with *Rs*7. Reaction of SM-PB1-9 (*chi11*) **A**, SM-PB1-5 (*tlp*) **B**, SM-PB1-1 (*chi11* + *tlp* + *Xa21*) **C**, and untransformed PB1 **D** to *Rs*7 infection at 24, 48, and 72 HAI

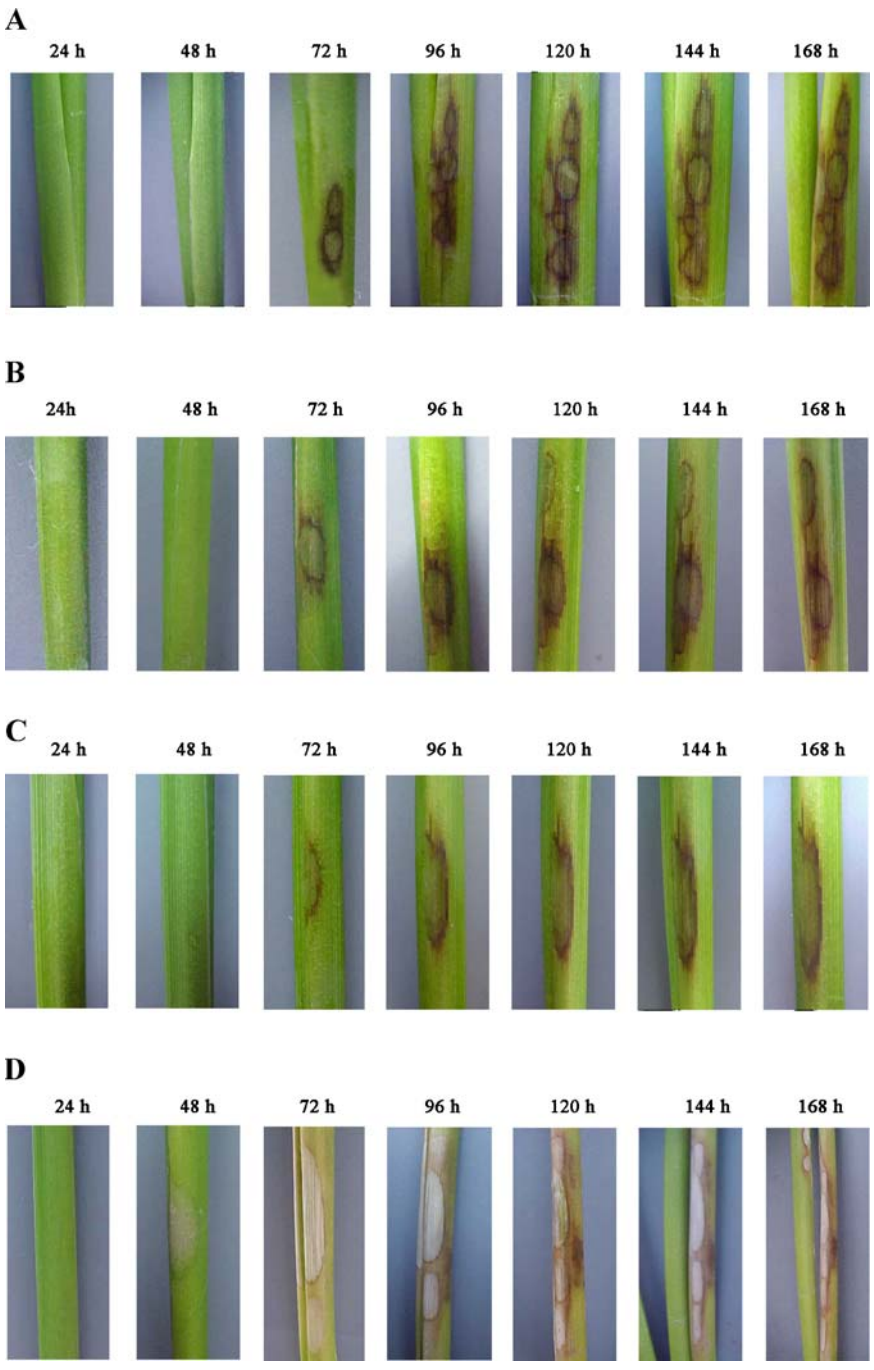

Fig. 9 Bioassay using leaf sheaths intact in the transgenic (T_1) PB1 lines with *Rs*7. Reaction of SM-PB1–9 (*chi11*) **A**, SM-PB1-5 (*tlp*) **B**, SM-PB1-1 (*chi11* + *tlp* + *Xa21*) **C**, and untransformed PB1 **D** to *Rs*7 infection at 24, 48, 72, 96, 120, 144, and 168 HAI

In the recent years, there are several successful reports on improved fungal disease resistance of crop plants by the expression of antifungal proteins like chitinases, glucanases, and TLPs (Broglie et al. 1991; Zhu et al. 1994; Lin et al. 1995; Datta et al. 1999; Anand et al. 2003; Kumar et al. 2003; Kalpana et al. 2006). Lin et al. (1995) reported development of lesions within 3–4 days after inoculation with *R. solani* on both untransformed and transgenic rice plants expressing rice chitinase (*chi11*) gene. However, they observed a reduction in number and size of lesions in the transgenic plants, when compared to untransformed controls. Though the lesions spread invasively throughout the leaf in control plants, they were restricted to the lower half of the leaf in transgenic plants. Datta et al. (1999) observed a mean sheath infection density of 8.2–19.1% and 36.6% in the transgenic rice plants expressing TLP and control plants, respectively. Similarly, potato plants expressing higher levels of osmotin-like protein (a member of the PR-5 group) were reported to confer an increased tolerance to the late blight fungus, *Phytophthora infestans* (Zhu et al. 1996).

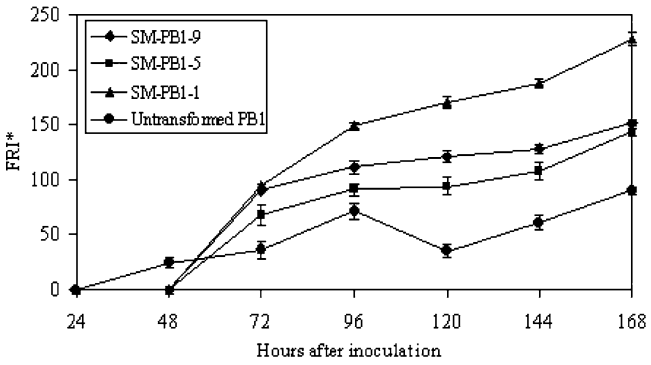

Fig. 10 Functional resistance/susceptibility index in transgenic (T_1) and nontransgenic PB1 lines upon inoculation with *Rs*7. *Each data point represents mean ± S.D of three replications (5 tillers/replication)

Transgenic coexpression of more than one PR-proteins could confer enhanced resistance than the expression of single PR-proteins. In the present study, it was observed that the transgenic line SM-PB1-1 expressing both *chi11* and *tlp* showed enhanced resistance to ShB pathogen than the lines expressing either of these genes (Fig. 8–10; Tables 3 and 4). Leah et al. (1991) demonstrated the synergistic antifungal activities of chitinases and β-1,3-glucanases under laboratory conditions. Zhu et al. (1994) studied the constitutive coexpression of a rice chitinase (*RCH10*) and an alfalfa acidic glucanase (*AGLU1*) in transgenic tobacco plants and reported that combination of the two PR-proteins enhanced significantly the protection against the fungal pathogen *Cercospora nicotianae*, causal agent of frogeye leafspot, than by individual transgenes. Similarly, evidence for synergistically enhanced protection in transgenic tomato (Jongedijk et al. 1995) and wheat (Anand et al. 2003) plants by the coexpression of chitinase and β-1,3-glucanase was reported. Kalpana et al. (2006) reported the co-expression of *chi11* and *tlp* in an indica rice line and the resultant enhanced ShB resistance. Enhanced resistance observed in the present transgenic lines coexpressing chitinase and TLP may be attributed to the different modes of action of these proteins. Chitinase degrades the fungal cell wall chitin, whereas TLP targets the fungal plasma membrane (Yun et al. 1998). These proteins could act synergistically (Hejgaard et al. 1991) to effectively kill the fungal pathogens. Moreover, ShB assays using detached leaves and leaf sheaths intact in T_2 progeny lines SM-PB1-1, 5, and 9 also evidenced stable inheritance of the resistance. There was no significant variation in the number of infection cushions, HRLH%, and total lesion spread between T_1 and T_2 progeny lines (Tables 3 and 4). The PB1 line expressing both *chi11* and *tlp* conferred better protection against ShB than the lines expressing individual genes.

Table 5 Bioassay of the transgenic (T_1 and T_2) lines for *Xanthomonas oryzae* pv. *oryzae* resistance

Line	Lesion length (cm)[a]		Disease reaction[b]
	T_1	T_2	
SM-PB1-1 (*chi11* + *tlp* + *Xa21*)	1.67 a	2.02 a	Resistant
Untransformed PB1	15.25 c	14.58 b	Susceptible
SM-ASD16-2 (*Xa21*)	1.40 a	1.21 a	Resistant
Untransformed ASD16	14.79 b	16.12 c	Susceptible

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

[a]Mean of five replications (5–10 leaves/replication).

[b]Lesion length 1–3 cm, resistant; 3–6 cm, moderately resistant; > 9 cm, susceptible (Mew and Vera Cruz 1979).

In bioassay for BB resistance, consequent to clip inoculation with *Xoo*, BB symptom appeared within 4–5 days after inoculation in both transgenic (SM-PB1-1 and SM-ASD16-2) and control (PB1 and ASD16) plants. Water-soaked lesion which developed from the cut surface spread along the veins of the inoculated leaf presenting a yellowish lesion with wavy margins. However, on 14 days after inoculation, lesion length of two transgenic T_1 lines SM-PB1-1 and SM-ASD16-2 were restricted to 1.67 and 1.40 cm, respectively, while in the untransformed PB1 and ASD16 plants the lesions attained a length of 15.25 and 14.79 cm, respectively (Table 5; Fig. 11A and B). Similar trend was observed in the bioassay performed on transgenic T_2 progeny lines expressing *Xa21* with BB pathogen, *Xoo* (Table 5). *Xa21* is a major dominant gene for rice BB resistance whose multi-isolate/race resistance potential against *Xoo* has been well established (Song et al. 1995; Wang et al. 1996). Tu et al. (1998) observed much reduced lesions of < 3.1 cm in transgenic BB-resistant plants and a *Xa21* donor (IRBB21), in comparison to the larger lesions of 13.3–20.3 cm in con-

Table 4 Assessment of sheath blighting in transgenic lines by bioassay using leaf sheaths intact

Line	HRLH (%)[a]		Total lesion spread (cm)[a]	
	T_1	T_2	T_1	T_2
SM-PB1-9 (*chi11*)	7.28 c	6.84 b	7.94 b	8.02 b
SM-PB1-5 (*tlp*)	5.97 b	6.02 b	8.04 b	9.82 c
SM-PB1-1 (*chi11* + *tlp* + *Xa21*)	2.13 a	2.45 a	4.90 a	3.87 a
Untransformed PB1	14.09 d	13.81 c	15.50 c	17.13 d

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

[a]Mean of five replications (5 tillers/replication).

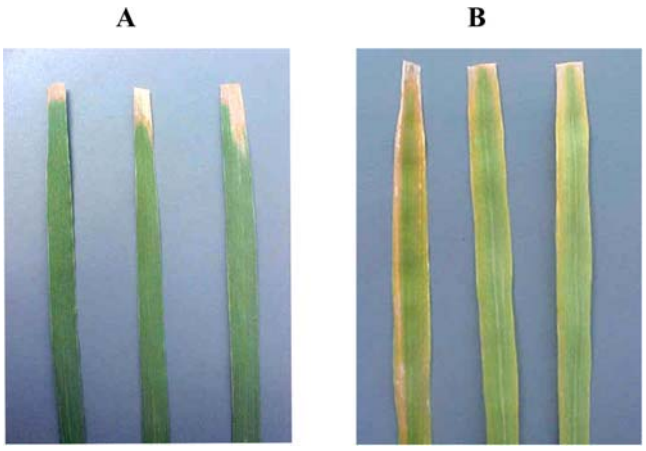

Fig. 11 Screening of transgenic (T_1) indica rice lines for bacterial blight resistance. Reaction of SM-PB1-1 (*chi11* + *tlp* + *Xa21*) **A** and untransformed PB1 **B** to *Xoo* infection at 14 days after inoculation

trol plants. Moreover, their *Xa21* transgenic IR72 lines performed well also under field conditions (Tu et al. 2000). As the pyramided line SM-PB1-1 did not segregate for *Xa21* in both T_1 and T_2 progenies, the influence of *chi11* and/or *tlp* on *Xa21*-mediated bacterial blight resistance could not be assessed. Previously, we developed several *Xa21* transgenic PB1 lines, the *Xoo* inoculation of which resulted in the induction of lesions (Ramesh et al. 2001) comparable in size to the ones exhibited by the presently pyramided line. Hence, at this point, the possible existence of any interaction between the defence (*chi11* and *tlp*) genes and the resistance (*Xa21*) gene could not be explained.

In summary, present study reports a successful incorporation of multiple disease resistance/defense genes in PB1, an indica rice cultivar, which led to an enhanced resistance to an agronomically important fungal pathogen and a bacterial pathogen. Transgenic lines obtained in this study add variability to the existing germplasm and thus paving way for an environmental-friendly means to achieve a better disease management. Intensification of research in these lines with a view to enriching the available germplasm is *sine quo non* for future rice breeding programmes that aim at introgressing multiple disease resistance in elite cultivars.

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