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Pyramiding insect and disease resistance in an elite indica rice cultivar asd16

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Abstract

Pyramiding transgenes of interest is one of the strategies to engineer multiple stress resistance in crop plants. Transgenic plants which stably express different genes can be hybridized to bring these genes together in one plant. Transgenic rice (*Oryza sativa* L. cv. ASD 16) plants harbouring genes *Xa21* (conferring bacterial blight resistance), *tlp* (conferring resistance to sheath blight), or *gna* (conferring resistance to brown planthopper) were used in hybridization experiments. Sexual hybridization was carried out in two different gene combinations: *Xa21* × *gna* and *tlp* × *gna*. Molecular analyses were carried out to confirm the presence of transgenes. In F_1 generation, lines harbouring either gene in each of the cross-combination were selected and forwarded to F_2 generation. The presence of genes in F_2 generation was confirmed by PCR, Southern blot hybridization, and Western blotting. The F_2 progeneis harbouring *Xa21* and *gna* exhibited resistance against bacterial blight and moderate resistance against brown planthopper. Similarly, the F_2 lines of *tlp* and *gna* combination provided resistance against sheath blight and moderate resistance against brown planthopper. The level of resistance observed in pyramided lines for insect or pathogens was comparable to the resistance observed in their parental lines. Our study shows that pyramiding genes by hybridization between transgenic plants could be one of the strategies to develop cultivars with multiple biotic stress resistances.

Additional key words: bacterial blight, brown planthopper, *gna*, *Oryza sativa*, sheath blight, *tlp*, *Xa21*.

Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population and is one of the three major food crops (Mohanty 2013). Biotic stresses including diseases, insects, and weeds are serious constraints to rice production. Among various diseases infecting rice, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (Uyeda and Ishiyama) Dowson and sheath blight (ShB) caused by *Rhizoctonia solani* Kuhn are serious ones. Brown planthopper (BPH) (*Nilaparvarta lugens* Stal.) is a major sucking pest of rice. As the demand for rice is on the increase, there is a need to improve the productivity of crop by managing factors that influence yield. Rice yield losses caused by BB in severe cases can reach up to 50 % when plants are infected at the maximum tillering stage (Datta *et al.* 2002). The *Xa21*, a dominant gene for resistance to BB was transferred from a wild species, *Oryza longistaminata,* to cultivar IR24 by back crossing (Khush *et al.* 1990). Ronald *et al.* (1992) isolated *Xa21* by positional cloning. Transfer of this gene into the genome of an elite indica rice cultivar IR72 and field evaluation has been carried out by Tu *et al.* (2000). The hybrid rice plants with *Xa21* displayed high broad spectrum resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) races besides maintaining agronomic performance (Zhai *et al.* 2001). BB resistant indica rice lines of ASD16, PB1, ADT38, and White Ponni engineered with *Xa21* following particle bombardment method of transformation were developed by our group (Maruthasalam *et al.* 2007). Baliyan *et al*.

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Abbreviations: BB - bacterial blight; ShB - sheath blight; BPH - brown planthopper; HRLH% - highest relative lesion height percentage; PR - pathogenesis related; TLP - thaumatin-like proteins; *Xoo* - *Xanthomonas oryzae* pv. *oryzae*
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(2016) demonstrated higher resistance against BB in pyramided rice genotype CSR-30 harbouring three genes (*xa5*, *xa13*, and *Xa21*) than in lines expressing single or two genes. Similarly, marker assisted transfer of *Xa21* and *Xa33* into an elite line of rice DRR17B showed high BB resistance without compromising the agro-morphological attributes of parental lines (Balachiranjeevi *et al*. 2018).

The ShB of rice caused by soil borne fungus *Rhizoctonia solani* Kuhn [teleomorph stage, *Thanatephorus cucumeris* (Frank) Donk], is a dreadful disease in many rice growing areas of the world. Yield loss ranges from 8 to 50 %, particularly when the infection is severe (Savary *et al.* 2000). Among different pathogenesis related (PR)-proteins, chitinases (PR-3 proteins) and thaumatin-like proteins (TLP; PR-5 proteins) have been employed effectively to engineer ShB resistance in elite rice cultivars, worldwide. Several TLPs were isolated and cloned from plant species including maize (Roberts and Selitrennikoff 1990), bean (Sehgal *et al.* 1991), sorghum, oat (Vigers *et al.* 1991), wheat (Rebmann *et al.* 1991), and rice (Velazhahan *et al.* 1998). Plants with high expression of TLP were shown to be resistant to the ShB pathogen (Datta *et al.* 1999). The indica rice cvs. PB1, ASD16, and White Ponni which were engineered with *tlp* in our laboratory through biolistic mode of transformation showed enhanced resistance against ShB pathogen, *R. solani* (Maruthasalam *et al.* 2007). In recent studies, combinations of defence genes always showed stronger and durable resistance to fungal and/or bacterial pathogen(s) than those lines expressing single gene (Fukuoka *et al*. 2015, Yasuda *et al*. 2015, Arunakumari *et al*. 2016). Gene pyramiding not only extends the durability of resistance but also enhances the spectrum of resistance.

Rice BPH (*Nilaparvarta lugens* Stal.) belonging to the order *Homoptera* is one of the major insect pests of rice causing huge crop loss (Khush *et al.* 1990). BPH causes direct physiological damage to rice by the removal of phloem sap and blockage of phloem vessels leading to hopper burn (Ou 1985). Plant lectins were shown to produce chronic effects on survival and development of insect pests belonging to different insect orders (Czapla and Lang 1990, Habibi *et al.* 1993, Powell *et al.* 1993, 1995). The snowdrop lectin (*Galanthus nivalis* agglutinin: GNA) is the most toxic of the lectins tested, decreasing BPH survival by 50 % at concentrations as low as 6 µM (Powell *et al.* 1995). Bioassays carried out in transgenic rice plants expressing *gna* indicated that the GNA is effective in decreasing survival, development and fecundity in BPH (Rao *et al.* 1998).

One of the major objectives of rice biotechnology programmes is to pyramid genes with a view to imparting multiple stress resistance. Only a few reports are available on transgenic lines carrying genes effective against more than one taxa of pathogen or insect pest. The simultaneous introduction of three insecticidal genes (the *Bt* genes, *cry1Ac*, and *cry2A* and the snowdrop lectin gene, *gna*) into commercially important indica rice cultivars by particle bombardment for broad spectrum resistance against different rice pests: leaffolder (*Cnaphalocrocis medinalis*) yellow stemborer (YSB, *Scirpophaga incertulas*) and BPH was reported by Maqbool *et al.* (2001). Transgene-pyramided stable rice lines resistant to disease and insect pests were developed by conventional crossing of two transgenic parental lines transformed independently with different genes. IR72 engineered with a rice chitinase (*RC7*) gene (for ShB resistance), *Xa21* (for BB resistance) and the *Bt* fusion gene (for insect resistance) were developed by reciprocal crossing of two transgenic homozygous IR72 lines (Datta *et al.* 2002). The BB resistance gene, *Xa21* and a *Bt* fusion gene *cry1Ab*/*cry1Ac* conferring insect resistance were pyramided into Minghui 63 by marker assisted selection. The pyramided line and its derived hybrids show high resistance against leaffolder, YSB, and BB (Jiang *et al.* 2004). Breeding of transgenic restorer line for multiple resistance against BB, striped stem borer and herbicide tolerance was developed by conventional crossing of two stable transgenic rice lines: Zhongguo91 with *cry1Ab* and *bar* genes and Yujing6 with *Xa21* gene (Wei *et al.* 2008). These approaches maximize the utility of gene transfer technology to introduce combination of different genes into rice providing a multi-mechanism defence.

Although several reports are available on rice transformation using single genes of interest, only a few reports are available on gene pyramiding in rice against both insect and disease. This urges the need for developing multiple resistance rice lines to effectively combat multiple biotic stresses. The present study was carried out to develop lines with multiple resistance to insect (BPH) and diseases (BB or ShB) in an elite local rice cultivar ASD16 by employing conventional crossing between transgenic events homozygous for transgenes.

Materials and methods

Generation of pyramided lines: Sexual hybridization of homozygous transgenic rice (*Oryza sativa* L.) lines were done in our laboratory earlier and X_1 and X_2 (two homozygous *Xa21* lines conferring BB resistance), T_1 and T_2 (two homozygous *tlp* lines governing ShB resistance) and G (a homozygous gna line expressing BPH resistance) were grown in the transgenic greenhouse at Tamil Nadu Agricultural University, Coimbatore, India.

The homozygosity was confirmed by PCR and Western blotting. Two different crosses, *Xa21* × *gna* and *tlp* × *gna* were performed. After the sexual hybridization, F_1 hybrid seeds were collected at maturity for further studies. F_1 progeny of the different cross-combinations were forwarded to F_2 generation after confirming expression of both the genes (*Xa21* and *gna* or *tlp* and *gna*). The resulting F_2 progenies were named X_1G and X_2G (*Xa21* × *gna*) and T_1G and T_2G (*tlp* × *gna*) according to the gene combinations.

Southern blot hybridization analysis: The parental homozygous ASD16 lines and their selected F_2 lines were analyzed by Southern blot hybridization. The genomic DNA (10 µg) extracted from transgenic plants and non-transgenic control plants and 5 ng of plasmid DNAs

(positive control) were digested overnight with appropriate restriction endonucleases (*Bangalore Genei*, Bangalore, India). The products were electrophoresed on 1.0 % (m/v) agarose gels and then transferred onto nylon membranes (*Hybond*$^{+}$, *Boehringer*, Mannheim, UK). The blots were subsequently hybridized overnight with radiolabelled probes prepared by random primer oligo labelling method (*Random Primer Labeling* kit, *Bangalore Genei*).

To confirm the presence of *Xa21*, the genomic DNA isolated from F_2 progenies and their parental lines 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. To confirm the presence of *tlp* gene, the genomic DNA 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. Similarly, the genomic DNA were digested with *Kpn*I to release the 480 bp *gna* expression cassette and blotted onto the membrane with a view to demonstrating the presence of *gna* gene. The blot was then probed with α-^{32}P-dCTP-labelled 5.0 kb fragment obtained by digesting p*ubi-gna* with *Kpn*I enzyme.

Bioassay experiments: Bioassays for BB and BPH were performed in two F_2 lines carrying both *Xa21* and *gna* genes. Similarly, bioassays for ShB and BPH were carried out in two F_2 lines harbouring both *tlp* and *gna* genes. In all the experiments, F_2 lines, parental homozygous lines, and non-transformed control plants were used. All the bioassay experiments were repeated thrice under the same conditions to confirm the resistance.

A virulent isolate of Xoo (Directorate of Rice Research, Hyderabad, India) maintained on peptone sucrose agar (PSA; 5.0 g dm^{-3} peptone, 20.0 g dm^{-3} sucrose, 0.5 g dm^{-3} $Ca(NO_3)_2 \cdot$. 4 H_2O, 0.5 g dm^{-3} $FeSO_4$. 7 H_2O, 2.0 g dm^{-3} Na_2HPO_4 .·2 H_2O, 20.0 g dm^{-3} 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 cm^3) into the 48-h-old culture and adjusting bacterial population to 10^9 cells cm^{-3}. All inoculations were made within 30 - 60 min after preparing the bacterial suspension.

The parental homozygous *Xa21* lines along with their F_2 derivatives (expressing *Xa21* and *gna*) 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-d-old plants at maximum tillering stage. After inoculation, the plants were covered with polyethylene bags with inner surface sprinkled with sterile distilled water to maintain plants under high relative humidity for better symptom development. From each line, five plants (5 - 10 leaves per plant) were inoculated along with suitable non-transgenic control plants. Lesion length was measured 14 d 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).

The parental homozygous *tlp* lines with their F_2 derivatives (expressing *tlp* and *gna*) were evaluated for ShB resistance along with suitable non-transgenic control plants. Two different ShB assay methods, one using detached leaves and another with intact leaf sheaths, were employed for screening the transgenic line, as described by Kumar *et al*. (2003). All the inoculations were carried out with 5 mm mycelial discs obtained from 3-d-old *Rs*7 (a virulent isolate of *R*. *solani*; Krishnamurthy *et al.* 1999) maintained on potato dextrose agar (250.0 g dm^{-3} potato, 20.0 g dm^{-3} dextrose, 20.0 g dm^{-3} agar, pH 7.0).

In bioassay using detached leaves, observations were made at 24-h intervals and infection cushions were counted under a stereo microscope, 72 h after inoculation (HAI). In bioassay using intact leaf sheaths, observations were made up to 7 d at 24-h intervals. At 168 HAI, the highest relative lesion height percentage (HRLH%) and the total lesion spread were estimated. HRLH% was calculated as: HRLH% = (length of the highest lesion / plant height) × 100.

For screening BPH resistance, standard seedling box screening test described by Heinrich and Mochida (1984) was followed. Parental transgenic homozygous *gna* line and four F_2 lines (expressing *Xa21* and *gna* or *tlp* and *gna*) along with a non-transgenic ASD16 control were used. Each transgenic line was sown in a row of the seed box (approximately 25 seeds/row). Simultaneously, one row of a susceptible check, TN1 and one row of a resistant check, PTB33 were also sown at random in seedling box. The BPH population cultured on TN1 plants was used to infest the seedlings. The seedlings were infested 14 d after sowing (DAS) with second and third instar nymphs. The plants with nymphs were gently tapped over the seedlings in such a way that approximately 4 - 5 nymphs settle on each seedling. Three replications (5 - 10 seedlings per replication) were maintained for each line. After the infestation, the lines were observed regularly for BPH damage symptoms. When the seedlings of the susceptible check were about 90 % dead, the test lines were examined and each seedling was given a score of 1 (= very slight damage) to 9 (= all plants dead) as per the damage scales of the Standard Evaluation System for rice (*IRRI* 1988).

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

Results

During Southern blotting analysis, *Eco*RV fragment of pC822, hybridization signal of 3.8 kbp was detected in the blots probed with the 3.8 kbp in parental lines, X_1, X_2, and F_2 lines, X_1G and X_2G. In addition to this, a high molecular mass band was observed in all the transgenic lines including non-transgenic control (Fig. 1). The banding pattern of parental lines and the corresponding

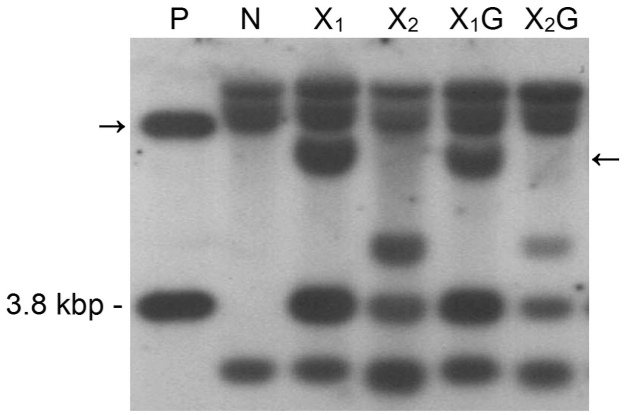

Fig. 1. Southern blotting analysis of the *Xa21* gene in parental ASD16 and F_2 lines. The 3.8 kbp band indicates the stable integration of the *Xa21* gene in their genomes, and *arrows* indicate possible hybridization to the polymorphic member of the *Xa21* gene family. *Lane P* - positive control (pC822 digested with *Eco*RV), *lane N* - non-transgenic control, *lanes* X_1 and *X2* - transgenic ASD16 lines harbouring the *Xa21* gene, *lanes* X_1G and X_2G - transgenic lines harbouring both *Xa21* and *gna* genes.

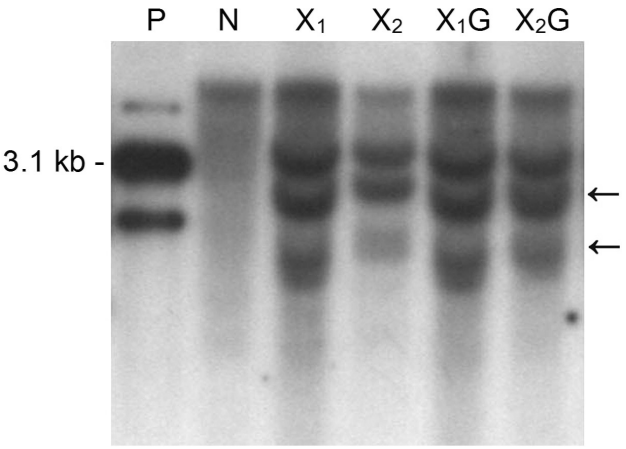

Fig. 2. Southern blotting analysis of *tlp* in parental ASD16 and F_2 lines. The 3.1 kbp band indicates the stable integration of TLP expression cassette (3.1 kbp) in their genomes, and *arrows* indicate possible hybridization to the endogene with sequence homology to the introduced (*tlp*) gene. *Lane P* - positive control (pGL2 digested with *Hin*DIII), *lane N* - non-transgenic control, *lanes* T_1 and *T2* - transgenic lines harbouring *tlp* gene, *lanes* T_1G and T_2G - transgenic lines harbouring both *tlp* and *gna* genes.

F_2 progeny lines were found to be similar indicating the stable integration and inheritance of *Xa21*. The blot with genomic DNA of F_2 progenies of lines harbouring *tlp* and *gna*, when probed with 1.1 kbp *tlp* coding sequence, showed a 3.1 kbp hybridization signal in two independent parental lines, T_1, T_2, and two F_2 lines, T_1G and T_2G (Fig. 2). When transgenic F_2 progenies harbouring *Xa21* and *gna* were subjected to Southern hybridization, α-^{32}P-dCTP-labeled *gna* detected a 480 bp band in the parental line G and corresponding F_2 lines, X_1G, X_2G, T_1G, and T_2G revealing the stable integration and inheritance of

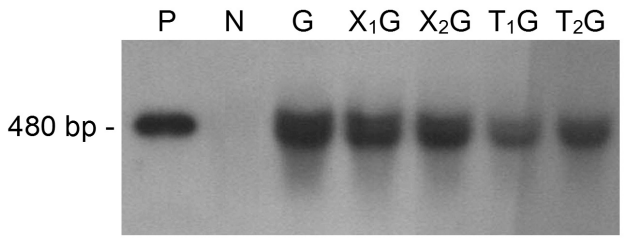

Fig. 3. Southern blotting analysis of *gna* in parental ASD16 and F_2 lines. The 480 bp band indicates the stable integration of *gna* expression cassette (3.1 kbp) in their genome. *Lane P* - positive control (p*ubi-gna* digested with *Kpn*I), *lane N* - non-transgenic control, *lane G* - transgenic ASD16 lines harbouring *gna* gene, *lanes* X_1G and X_2G - transgenic lines harbouring both *Xa21* and *gna* genes, *lanes* T_1G *and* T_2G - transgenic lines harbouring both *tlp* and *gna* genes.

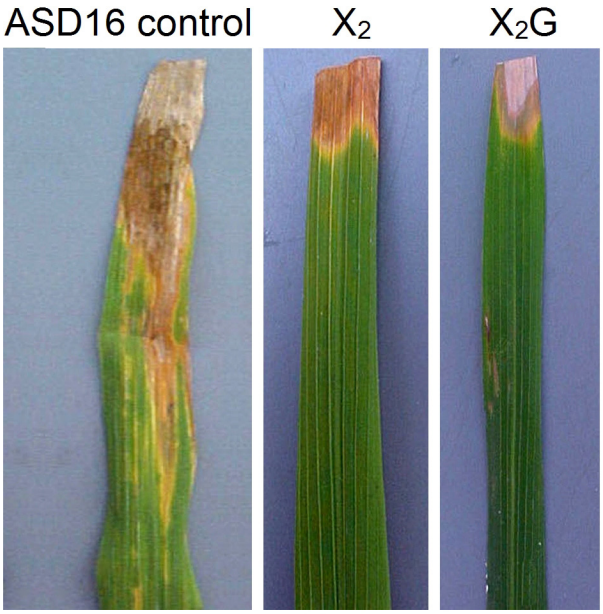

Fig. 4. Assessment of bacterial blight resistance by leaf clipping method in the parental ASD16 line and its F_2 progeny expressing the *Xa21* gene.

gna expression cassette in their genomes (Fig. 3).

The BB symptom initiated within 4 - 5 d after inoculation in both transgenic (parental lines and F_2 lines) and ASD16 control plants. Initially water-soaked lesions developed from the cut surface which spread along the veins of the inoculated leaf presenting a yellowish lesion with wavy margins (Fig. 4). However, on day 14 after inoculation, lesion length on non-transgenic ASD16 plants registered 17.07 cm, whereas in one of the parental lines, X_2 and it's F_2 progeny, X_2G, the lesion length was restricted to 2.31 and 2.55 cm, respectively (Fig. 4, Table 1). An another parental line, X_1 and it's F_2 progeny, X_1G also recorded relatively lesser lesion length of 2.80 and 2.87 cm respectively (Table 1).

In bioassay of ShB using detached leaves, the pathogen induced formation of infection cushions away from the

Table 1. Bioassay of parental *Xa21* lines and their F_2 progenies for resistance to *Xanthomonas oryzae* pv. *oryzae*. Means of five replications (5-10 leaves/replication). Lesion lengths: 1 - 3 cm (resistant), 3 - 6 cm (moderately resistant), >9 cm (susceptible). Means followed by different letters are significantly different at 5 % level by the Duncan's multiple range test.

Line	Lesion length [cm]	Disease reaction
X_1 (parent)	2.80^c	resistant
X_2 (parent)	2.31^a	resistant
X_1G	2.87^d	resistant
X_2G	2.55^b	resistant
ASD16 control	17.07^e	susceptible

Table 2. Assessment of sheath blight resistance in parental *tlp* lines and their F_2 progenies by detached leaf assay. Means of three replications (three leaf bits/replication). Means followed by different letters are significantly different at 5 % level by the Duncan's multiple range test.

Line	Number of infection cushions
T_1 (parent)	15.20^a
T_2 (parent)	16.40^c
T_1G	15.90^b
T_2G	16.90^d
ASD16 control	47.80^e

site of inoculation by putting forth new hyphae occurred 48 HAI in both transgenic and non-transgenic plants. However, the frequency of formation of infection cushions in non-transgenic ASD16 was 47.8, while it was only 15.2 in T_1, 16.4 in T_2, 15.9 in T_1G, and 16.9 in T_2G (Table 2). Moreover, in all transgenic lines, small brownish lesions (3 - 5 mm diameter) were formed at the site of infection cushion formation at 48 - 72 HAI, while the inoculated leaves remained green and fresh. However, in non-transgenic ASD16 leaves, lesions (1.5 - 2.0 cm) with brown margins and grey centre were formed at the site of infection cushion formation. These lesions gradually enlarged in size covering almost the entire length of the leaf

Table 3. Assessment of sheath blighting using highest relative lesion height percentage (HRLH%) and total lesion spread in the parental *tlp* lines and their F_2 progenies. Means of three replications (three tillers/replication). Means followed by different letters are significantly different at 5 % level by the Duncan's multiple range test.

Line	HRLH%	Total lesion spread [cm]
SM-ASD16-1-3-2 (parent)	2.34^b	4.91^b
KL-ASD16-2-1-5 (parent)	2.66^c	6.74^d
T_1G	2.15^a	4.47^a
T_2G	2.76^d	6.25^c
ASD16 control	8.40^e	11.83^e

Table 4. Bioassay of the F_2 progenies of *gna* for brown planthopper resistance. Means of three replications (5 - 10 seedlings per replication). Damage rating: 1 - 3 (resistant), 3 - 5 (moderately resistant), 5 - 7 (susceptible), 7 - 9 (highly susceptible). Means followed by different lettesr are significantly different at 5 % level by the Duncan's multiple range test.

Line	Damage rating	Insect reaction
G	4.90^e	moderately resistant
X_1G	4.40^b	moderately resistant
X_2G	4.80^d	moderately resistant
T_1G	4.70^c	moderately resistant
T_2G	5.60^f	susceptible
ASD16 control	7.70^g	highly susceptible
PTB33 (resistant check)	2.80^a	resistant
TN1 (susceptible check)	8.60^h	highly susceptible

leading to yellowing and drying within 72 HAI (Fig. 5).

In intact leaf sheath assay, the HRLH% and total lesion spread were the factors used to study the relative resistance/susceptibility of transgenic and non-transgenic lines for sheath blighting. In transgenic parental and F_2 lines, no lesions appeared at the site of inoculation till 48 HAI, whereas in non-transgenic ASD16 plants, a blanch lesion with a thin brown border was formed within 48 HAI (Fig. 6). These lesions slowly turned into grey

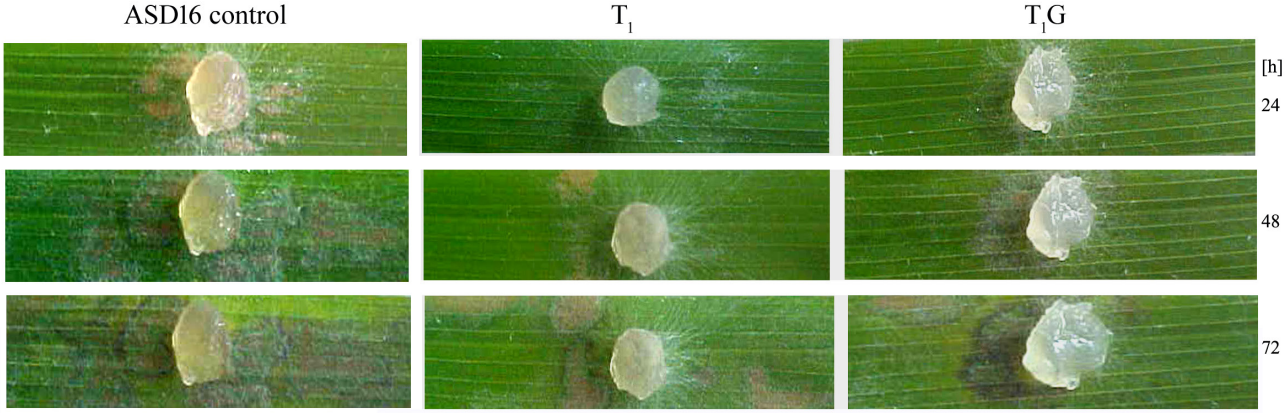

Fig. 5. Assessment of sheath blight resistance by detached leaf assay in the parental ASD16 line and its F_2 progeny expressing *tlp*.

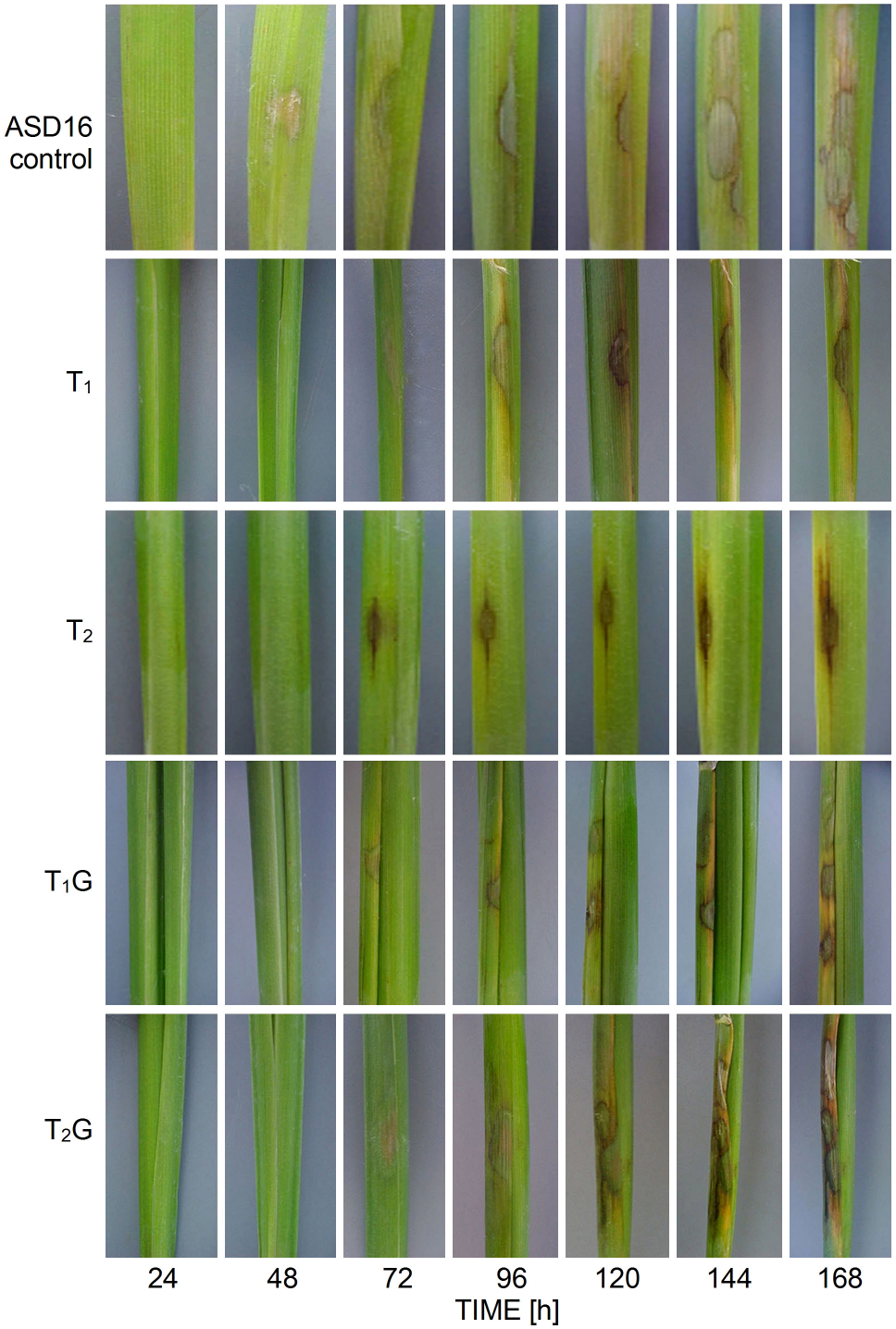

Fig. 6. Assessment of sheath blight resistance by intact leaf sheath assay in the parental ASD16 line and its F_2 progeny expressing *tlp*.

and enlarged in size covering the entire leaf sheath within 168 HAI. Contrastingly, in all the transgenic parental lines (T_1 and T_2) and their F_2 lines expressing *tlp*, small brownish lesions appeared at 72 HAI, the lesion spread was very much restricted and an extensive browning of area surrounding the lesions were noticed (Fig. 6). In addition, the pathogen spread over the entire leaf sheaths, leading to complete drying of infected leaf sheath in non-transgenic plants within 168 HAI and such a drying was absent in transgenic lines expressing *tlp*.

The HRLH of T_1, T_2, T_1G, and T_2G was recorded as 2.34, 2.66, 2.15, and 2.76 % respectively, whereas it was 8.4 % in control plants (Table 3). Total lesion spread on leaf sheath of the transgenic F_2 line, T_1G (expressing *tlp* and *gna*) after 168 h of inoculation was 4.47 cm as compared to 6.25 cm in T_2G (expressing *tlp* and *gna*), while total lesion

Fig. 7. Assessment of brown planthopper resistance by seedling box screening test in the parental ASD16 line and its F_2 progeny expressing *gna*.

spread on non-transgenic ASD16 was 11.83 cm (Table 3). Similarly, the parental homozygous lines T_1 (4.91 cm) and T_2 (6.74 cm) showed a significant reduction in the total lesion spread compared to control (Table 3).

A homozygous *gna* parental line, G and F_2 progenies were evaluated for their resistance against BPH. First and second leaves with orange tips and slight stunting symptom initiated 3 d after insect release in non-transgenic ASD16 control and susceptible check (TN1). The similar type of symptom initiation in transgenic plants and resistant check PTB 33 was observed only on 6 d after the insect release. Severe stunting, drying, and mortality of seedlings in TN1 were noticed on 9 d after the insect release with a mean damage grade of 8.6 (Fig. 7, Table 4). The scoring was done only after TN1 exhibited more than 90 % damage which normally noticed 9 d after the insect release. Of the transgenic lines and controls tested, PTB 33, the resistant check scored the lowest mean damage rating (2.80), while transgenic parental line and it's three F_2 lines exhibited a damage rating between 4.4 and 4.9. The F_2 lines, X_1G, T_1G, X_2G and their parental line, G registered a mean damage rating of 4.4, 4.7, 4.8, and 4.9 respectively. However, these mean damage rating were significantly lesser than in non-transgenic control which showed a mean damage rating of 7.7 (Table 4).

Discussion

Though the management of biotic constraints such as, BB, ShB, and BPH has been achieved through use of synthetic pesticides, it is neither sustainable nor environmentally safe. An effective alternate to chemical control is the exploitation of host plant resistance. Developing biotic stress resistant crop cultivars through conventional breeding methods is time consuming. However, genetic engineering to develop insect and pathogen resistant lines as a part of plant breeding programme could overcome problems posed by conventional methods of controlling pest and diseases.

In the present study, we pyramided two transgenes in an elite indica rice cultivar ASD16 to develop lines expressing combinations of traits by conventional crossing of transgenic homozygous lines. Sexual hybridizations were performed in two different combinations in order to get two genes in a pyramided line. The pyramided F_2 lines showed enhanced protection against BB or ShB and BPH. Datta *et al.* (2002) pyramided three transgenes *Xa21*, *Bt* (*cry*) and *RC7 chitinase* in a single elite rice line by conventional crossing of two IR72 transgenic homozygous stable lines, one carrying the *Xa21* gene and the other carrying both *Bt* and rice chitinase (*RC7)* genes in order to incorporate multiple resistance against BB, YSB, and ShB. It is important to ensure that the transgenic parents used for gene pyramiding through sexual crossing are homozygous lines for the individual resistance genes. Datta *et al.* (2002) used stable homozygous IR72 lines of *Xa21*, rice chitinase (*RC7*), and *Bt* (*cry*) for sexual crossing. In our experiments, homozygous lines for BB, ShB, and BPH resistance sources were used as parental lines in the hybridization programmes. Southern blot hybridization analyses demonstrated the presence of transgenes in parental lines as well as in F_2 progenies of all combinations.

Two transgenic lines, expressing *Xa21* and their F_2 derivatives exhibited resistance to *Xoo* with a lesion length of <3.0 cm, while non-transgenic ASD16 control was associated with a significantly higher lesion length of 17.07 cm. Wang *et al*. (1996) demonstrated resistance to multiple *Xoo* races in the transgenic lines harbouring *Xa21*. Tu *et al*. (1998) observed a lesion length of <3.1 cm in the BB-resistant plants and an *Xa21* donor (IRBB21) whereas non-transgenic control plants exhibited lesion lengths of 13.3 to 20.3 cm. Maruthasalam *et al*. (2007) reported similar spectrum of resistance in ASD16 (expressing Xa21) and Pusa Basmati1 lines (expressing *Xa21*, *chi11*, and *tlp*).

The ShB resistance in the homozygous parental lines and their F_2 generations were evaluated using two functional expression assay systems (Kumar *et al*. 2003). Present study using detached leaves revealed that the leaves of transgenic plants exhibited characteristic browning around the lesions signifying effective restriction of pathogenic invasion. Rapid yellowing and drying observed in the leaves of non-transgenic control was absent in the transgenic plants. Moreover, the number of infection cushions formed on leaves was

significantly lesser in transgenic lines expressing *tlp* and their F_2 derivatives as compared to non-transgenic control. Similar kinds of results were obtained in detached leaf assays by earlier workers (Kumar *et al*. 2003, Kalpana *et al*. 2006, Maruthasalam *et al*. 2007, Rajesh *et al*. 2016).

In intact leaf sheath assay, 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. In non-transgenic control plants, this browning was absent and drying of leaf sheaths was also noticed, while leaf sheaths of transgenic plants expressing *tlp* and their F_2 derivatives remained green, despite ShB infection. This clearly demonstrated that the pathogen spread was very much restricted in transgenic lines possibly due to an extensive browning at the site inoculation, a defense response very similar to hypersensitive reaction. Groth and Nowick (1992) suggested that resistance to the spread of *Rhizoctonia solani* in rice could be consequent to production of oxidised phenolics (a dark zone around lesions). Maruthasalam *et al*. (2007) reported the same pattern of resistance in transgenic Pusa Basmati lines carrying the *tlp* gene.

Transgenic ASD16 plants expressing *tlp* and their F_2 lines recorded significantly lesser HRLH% and total lesion spread as compared to control. There have been several reports of improved resistance of plants to diseases as a result of over expression of chitinases and TLPs (Brogue *et al.* 1991, Zhu *et al.* 1996, Lin *et al.* 1995, Datta *et al.* 1999, Datta *et al.* 2000). Lin *et al.* (1995) reported the development of lesions within 3—4 d after inoculation with *R. solani* on both non-transgenic and transgenic rice plants expressing rice chitinase (*chi*11) gene. However, the number and size of lesions in the transgenic plants were reported to be much smaller as compared to the control. The lesions invaded the upper half of the leaf sheaths in control plants, whereas in the transgenic plants the lesions were confined to the lower half. Datta *et al.* (1999) observed a mean sheath infection density of 8.2 - 19.1 % in transgenic rice plants expressing TLP against 36.6 % in control plants. In earlier reports, significantly lesser HRLH% and total lesion spread in transgenic lines expressing *chi11* and/or *tlp* were reported (Kumar *et al*. 2003, Kalpana *et al.* 2006, Maruthasalam *et al*. 2007). Recently, Rajesh *et al*. (2016) has demonstrated the inheritance of sheath blight resistance (upto T_4 generation) in transgenic ASD16 rice plants expressing a chitinase gene.

In the standard seedling box method of BPH screening when all the seedlings of TN1 were completely killed, each seedling of the transgenic and non-transgenic lines were examined and given a score from 0 to 9 based on the damage level, following Xu (2013). The development of stunting symptom was noticed very slowly in transgenic plants compared to TN1 and non-transgenic ASD16 plants. The parental *gna* line and its four F_2 derivatives exhibited moderately resistant reaction to BPH. Resistance rating was done only in the seedling stage, as varieties resistant at seedling stage were reported to be resistant at later stages of plant growth. This finding is in accordance with the report of Bharathi and Chelliah (1991). Rao *et al*. (1998) conducted BPH bioassays in transgenic rice plants expressing GNA and reported that expression of GNA in the transgenic plants resulted in reduction in survival and overall fecundity of the insects, retarded insect development and expressed a deterrent effect on BPH feeding. Maqbool *et al*. (2001) reported 25 % reduction in the survival of the BPH in the pyramided lines expressing *cry1Ac*, *cry2A* and *gna*, in addition, observed a very slow rate of insect development on *gna* expressing plants as compared to non-transgenic plants. Myint *et al.* (2012) reported a higher mortality (82.9 %) of female BPH in preliminary pyramided lines of ADR52 rice harboring two BPH resistance gene loci, namely BPH25 and BPH26, whereas a lesser percentage (11.4—14.3%) of female BPH mortality was recorded in preliminary near-isogenic ADR52 rice carrying either BPH25 or BPH26. Xu (2013) also got a similar kind of results for the pyramided japonica rice lines expressing BPH resistance genes, *BPH14* and *BPH15*.

The present study reports a successful pyramiding of insect and disease resistance genes in ASD16, an indica rice cultivar. The pyramided lines exhibited resistance to economically important bacterial or fungal pathogen and an insect pest. The expressions of genes in the parental lines and the respective F_2 lines were similar in terms of protection conferred by them. The pyramided lines that are identified in the present study could well be utilized in future breeding programmes which aim for enhanced resistance against BPH and diseases of rice.

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