Transgenic Rice Lines Expressing Rice Endochitinase (*Chi11*) Confers Improved Resistance to Sheath Blight and Rice Blast Fungal Pathogens

MANUBOTULA DURGA SAHITHYA[1], MOTE SRINATH[2], MOODAVATH SWATHI BAI[3], CHITTALA SIRISHA[4], KETHAVATH SRINIVAS NAIK[5]

[1, 2, 3, 5] *Centre for Plant Molecular Biology, Osmania University, Hyderabad, Telangana, India*

[4] *Loyola Academy, Degree & P.G. College, Hyderabad, Telangana, India*

Abstract*— Rice is the most important staple food crop for more than half of the world population. The global production needs to be doubled to meet the demand. But the major challenge is the increased severe crop loss due to insects and diseases. Among which rice blast caused by Magnaporthe grisea and sheath blight caused by Rhizoctonia solani, is considered are the most devastating diseases in different rice-growing regions of the world. Most of the productive high quality rice varieties presently cultivated in India lack durable resistance to major fungal and bacterial pathogens, such as, M. grisea and R. solani. In the present study, rice endochitinase (Chi11) has been introduced into commercial indica rice variety by Agrobacterium-mediated genetic transformation. Transgenic rice plants were regenerated from the phosphinothricin (PPT) - resistant calli obtained after co-cultivation with Agrobacterium strain LBA4404 harbouring Ti plasmid pSB111-bar-Chi11. Molecular analyses confirmed the stable integration and expression of Chi11 in various transgenic rice lines. Transgenes Chi11 and bar were stably inherited and disclosed co-segregation in T_1 generation in a Mendelian fashion. Segregation analysis of T_2 progenies using PPT test disclosed monogenic (1:2:1) genotypic inheritance of transgenes. Different Chi11 transgenic lines exhibited ~ 14 to 43 fold higher specific activity of endochitinase than that of untransformed control plants. Homozygous transgenic rice lines expressing CHI11 protein displayed enhanced resistance to rice blast and sheath blight diseases. This is the first report of its kind dealing with the transgenic indica rice exhibiting high resistance to two major fungal pathogens. These transgenic rice lines appear promising and might serve as a novel genetic resource in rice breeding aimed at durable resistance against various pathogens.

Indexed Terms*-- Agrobacterium-mediated transformation, Chi11 endochitinase, Rice blast, Sheath blast, Transgenic rice

I. INTRODUCTION

Rice is one of the most important staple food crops of the world, and more than half of the global population depends on it as a main source of nutrition. Owing to its small genome size (~389 Mb), ease of *Agrobacterium*-mediated transformation [1], [2] and genetic synteny with other cereal genomes [3], rice serves not only as a typical monocotyledon for research, but also as a model crop for genomics and evolutionary studies. In recent times, rice yield showed quantum leaps, mainly as a result of genetic improvement involving increased harvest index through deployment of DGWG semi-dwarf gene [4]-[6] and exploitation of heterosis by way of hybrid varieties [7]-[9] Currently, each hectare of agricultural land in Asia produces food for 27 people, but it needs to feed 43 people by 2050; thus the global rice production needs to be doubled [10]. It is essential to increase the productivity of the rice through improvements in cultivation practices, introduction of high-yielding varieties and hybrids.

However, a number of challenges have to be met to achieve the goal of increased rice production in a sustainable manner. The first challenge is the increasingly severe occurrence of insects and diseases in almost all the rice-producing areas, resulting in greater yield losses [11]. More than 70 diseases caused

by fungi, bacteria, viruses or nematodes have been recorded on rice. Among which rice blast caused by *Magnaporthe grisea* and sheath blight caused by *Rhizoctonia solani*, is considered as the most devastating diseases in different rice-growing regions of the world [12], [13]. Protection strategies safeguard nearly 38% of attainable rice production from pests [14] -[17]. In their absence, annual yield losses caused by the rice blast fungus alone varied from 10 to 30% of the harvest. But even 10% yield loss is significant which is sufficient to feed ~ 60 million people for one year [18]. In severe epidemics, yield losses due to sheath blight disease ranging from 8 to 50% have been reported [19], [20].

In most cases, applications of protective agrochemicals are not adequate to control rice diseases. Furthermore, the use of chemicals is subject to increasing restrictions because of their potentially harmful impact on the environment, and hence the control of diseases is often limited to prophylaxy [21]. However, the existence of natural sources of resistance to diseases has enabled the selection of a number of resistant varieties through conventional breeding. Classical hybridization is obviously restricted to within species (or between closely related species); while potentially effective resistance mechanisms may also exist elsewhere. In addition, it may be difficult and time consuming to introduce resistance from a wild species into commercial cultivars. However, recent improvements in plant-transformation techniques and progress in the understanding of plant-pathogen interactions enable the use of genetic engineering for the rational development of disease resistant plants. Unlike classical breeding, genetic engineering allows the modification or introduction of one or more resistant genes into susceptible varieties [22] –[23].

PR proteins were first described in the 1970s by Van Loon and Van Kammen, who observed accumulation of various novel proteins after infection of tobacco with Tobacco Mosaic Virus. Several PRs including PR-1, β-1, 3-glucanases (PR-2), chitinases (PR-3), PR-4 and osmotin (PR-5) revealed antimicrobial activities *in vitro* [24] – [26]. Moreover, chitinases and β -1, 3-glucanases exhibited a synergistic antifungal activities [27] –[29]. They also release various molecules that probably acted as elicitors [30] – [32]. Among PR-proteins, chitinases belonging to the PR-3 group appear to be potential candidates for management of fungal diseases [33]. Chitinases are hydrolytic enzymes that degrade the chitin-containing fungal cell wall, and result in the production of fungal elicitors that induce defense responses in plants [34], [35]. Endochitinases (EC 3.2.1.14) are the most extensively studied plant chitinases, which randomly hydrolyse internal β -1, 4-linkages of N-Acetyl-D-glucosamine (GlcNAc) polymer of the chitin, a major component of many fungal cell walls [36]. Plants synthesize various chitinases [37]. However, the endogenous substrate for plant chitinases has not yet been found. Whereas, in fungi, chitin was found to constitutes between 3% and 60% of the cell walls [38]. In the absence of an endogenous substrate, plant chitinases may be involved in the interaction between plants and microbes, which produce chitin and chitin-related compounds.

The rice endochitinase gene has been transferred successfully through genetic engineering into rice. The transgenic rice plants were found to have increased resistance to sheath blight disease and the level of resistance was positively associated with the amount of chitinase enzyme produced in the transgenics [39]-[43] and various other crop plants including tobacco [44], Melon [45], pea nut [46], canola [47], Maize [48], straw berry [49], cucumber [50], tobacco and tomato [51].

Most of the productive high quality rice varieties presently cultivated in India lack durable resistance to major fungal and bacterial pathogens, such as, *M. grisea* and *R. solani*. Broad-spectrum durable disease resistance is a long-term goal of crop protection, especially in developing countries, where control measures for many diseases are limited [52]. Significant advances thus far made in the regeneration protocols, and gene delivery methods of rice have facilitated the introduction of beneficial genes for various agronomic traits. In the recent past, it has been established that *Agrobacterium*-mediated transformation is an efficacious method for transferring novel candidate genes into elite *indica* rice varieties [53] -[55].

The present investigation deals with the construction of super binary vectors containing *Chi11* plant

expression cassette and its expression in elite indica rice cultivar using *Agrobacterium*- mediated genetic transformation method. Molecular evidences suggest stable integration of *Chi11* and *bar* genes into the genomes of rice plants, and their variable expression at RNA and protein levels. The stable transgenic lines, expressing CHI11 showed explicit resistance against major fungal diseases, viz., rice blast and Sheath blight

II. MATERIALS AND METHODS

A. Construction of Ti-super binary vector containing *Chi11* and *bar* expression cassettes

Rice endochitinase (*Chi11*) expression cassette was excised from *Hind* III site of pAHG11 vector, and cloned at *Hind* III site of pSB11 *bar* intermediate vector [56]. The binary vector contains *bar* (CaMV35S-*bar*-*nos*) gene serve as a plant selection marker [57]. The recombinant vector pSB11-*bar*-*Chi11* was maintained in HB101 cells and mobilized into *A. tumefaciens* strain LBA4404 by triparental mating using the helper vector pRK2013 and the resulting co-integrate vectors were designated as pSB111-*bar*-*Chi1* [58].

B. *Agrobacterium*-mediated transformation and regeneration of transgenic plants

Agrobacterium-mediated genetic transformation experiments were carried out using LBA4404 strain harboring pSB111-*bar*- *Chi11* super-binary vector. Elite indica rice cultivar Swarna (MTU7029), obtained from the Directorate of Rice Research (DRR), Hyderabad, was employed for genetic transformation. Mature seeds were manually dehusked and surface-sterilized with 0.1% (w/v) HgCl2 for 7 min followed by three washings with autoclaved distilled water, and kept at 29°C for germination. After 24 h of incubation, sprouted embryos were cut aseptically and placed on MS medium (3MN62; MS basal + 30 g/l maltose + 2 mg/l 2, 4-D + 1 g/l cas aminoacids +50 mg/l tryptophan +100 mg/l Inositol + 0.3% gelrite) for callus induction. After 3 weeks of incubation, the scutellum- derived calli were used for transformation experiments. *Agrobacterium* cultures were initiated by inoculating a single colony of the bacterium into 6 ml YEP medium containing 50 mg/l spectinomycin and 10 mg/l tetracycline at 225 rpm and 29°C for 24 h. The bacterial culture was pelleted at 3500 rpm and resuspended in 10 ml of PIMII medium [59] supplemented with 100 μM acetosyringone, and incubated for 16 h at 29°C. Before co-cultivation, the embryogenic calli were cut into small pieces, and were treated with MS basal medium supplemented with 100 mM acetosyringone for 30 min. Later, calli were transferred into the *Agrobacterium* culture and left on the shaker at 225 rpm for 30 min. These calli were placed on the co-cultivation medium and 20 μl of *Agrobacterium* culture was added on each callus for infection. Infected calli were incubated for 72 h at 29°C in dark and washed thrice in MS basal supplemented with 250 mg/l cefotaxime, and kept in 3MN62 medium containing the above antibiotics for 2 weeks. Proliferated calli were subjected to two rounds of selection containing 8 mg/l and 10 mg/l phosphinothricin for four weeks. After 4 weeks of incubation on selection medium, the surviving calli was selected and cultured on the proliferation medium for 2 weeks. Later, actively growing calli was transferred to the regeneration medium containing BAP (3–4 mg/l) and NAA (0.1–0.5 mg/l). Subsequently, the regenerated shoots were transferred onto the 1/2 MS rooting medium, and rooted plants were transferred into pots and grown to maturity in the glass-house. Transgenic plants (30–40 day old) along with untransformed controls were tested for their tolerance to the herbicide BASTA.

C. Southern blot analysis

Genomic DNA was isolated from the BASTA tolerant and untransformed control plants according to Japelaghi et al [60]. PCR analysis was carried out using the primers corresponding to the plant expression cassette of *Chi11* (CaMV F, 5´- GGA GCA TCG TGG AAA AAG AA-3´ and *Chi11* R, 5´- CCG TTG TTC TCC TCC TTG AA - 3´) and *bar* (*bar* F, 5´- CTA CCA TGA GCC CAG AAC G - 3´ and *nos* R, 5´- GTT TGC GCG CTA TAT TTT GTT -3´). The DNA from the untransformed control plants was used as negative control and the intermediate vector was used as positive control. For Southern blot analysis [61], approximately 10– 12 μg of genomic DNA was digested with *Eco*RI and *Hind*III separately, electrophoresed on a 0.8% agarose gel and subsequently transferred to an N+ Nylon membrane and fixed by exposing to UV (1200 μJ for 60 s) in an UV cross linker. DNA blot was pre-hybridized with sodium phosphate buffer (pH 7.2) containing 7% SDS and blocking reagent (Salmon sperm DNA) at 65°C

for 6 h. Hybridization was carried out with the same buffer at 65°C for 18–20 h. The 956bp *Chi11l* and 560 bp *bar* coding regions were used as probes after labelling with α-^{32}P dCTP employing ready to go random primer DNA labelling kit (Amersham Biosciences). The membrane was washed at room temperature (37°C) twice in buffer 1 (2 × SSC+0.1%SDS) for 20 min each, followed by once in buffer 2 (1 × SSC+0.1% SDS) for 15 min at 65°C and once in buffer 3 (0.1 × SSC+0.1%SDS) for 10 min at 65°C. Later, membranes were exposed to X-ray film for 24–48 h at -70°C.

D. Northern blot analysis

Northern blot analysis was carried out according to Sambrook and Russell [61]. About 20 µg of total RNA was separated on 1.4% denaturing agarose gel and was blotted onto nylon membrane and fixed by exposing to UV (1200 µJ for 60 s) in an UV cross linker. Pre-hybridization, hybridization and washing steps were carried out as described above for Southern blot analysis.

E. Assay for endochitinase activity

Endochitinase activity was detected and quantified using the procedure of Bolar *et al* [62] with certain modifications. Leaf tissue was ground in assay buffer consisting of 1 ml 10% SDS, 1 ml 10% Triton X-100, 2 ml 0.5 M sodium EDTA, 70 µl 14.4 M mercaptoethanol and 96 ml 100 mM sodium acetate buffer, pH 5.0. Ground leaf extract (100 µl) was mixed with 40 µl of substrate 4-methylumbelliferyl-β-D-*N*, *N'*, *N*''- triacetylchitotrioside (1 mg of substrate dissolved in 3.5 ml of 100 mM sodium acetate buffer at pH 5.0). The reaction was stopped at 15-min intervals with 0.2 M sodium carbonate and fluorescence was determined at 360/460 (excitation/emission) with FP-750 fluorescence spectrometer as recommended by the manufacturer (JASCO). Fluorescence of known concentrations of 4-methylumbelliferone (Sigma) was used to plot a standard curve to determine the activity of endochitinase present in the samples tested. Amount of protein in the leaf sample was determined using Bradford dye-binding protein assay as recommended by the manufacturer (Bangalore Genei). The activity of endochitinase was defined as nanomoles product released per minute per milligram of protein (nmoles/ min /mg).

F. Fungal bioassays

Fungal pieces of rice blast and Sheath blight diseases were carried out on both T_1 and T_2 *Chi11* transgenic plants along with their respective controls as well as susceptible control plants at the Directorate of Rice Research, (DRR), Rajendranagar, Hyderabad. Since the rice blast bioassays *Chi11* transforms along with untransformed controls, HR12 (susceptible) and IR64 (resistant) controls were germinated in seedling beds. Fifteen-day old seedlings were subjected to *M. grisea* strain IC9 (International Race C, Group 9, raised from a single-spore culture; origin DRR, Hyderabad) infection either by spraying spore suspension or by infecting with fully infected HR12 rice leaves. Day temperature during the infection season was about 28 ±2ºC at 85% relative humidity and night temperature was about 25±2ºC, ~90% relative humidity. Artificial sprinklers were used to maintain high relative humidity during day time and during nights the beds were covered with polythene paper pre-wetted with water. Response of the infected plants was scored within 10 days after infection using the SES scale (1996) based on length and type of lesions, percentage of disease spread and overall performance of seedlings [63]. For Sheath blight bioassays hypha from the pure culture of rice sheath blight pathogen, *R. solani*, was inoculated on potato dextrose agar (PDA) medium. After 3-days of incubation in the dark at 28±2ºC, a colonized piece of agar (2mm^2) was subcultured for another 3 days on PDA plate under the identical conditions. Shoots of typha were cut into pieces of 5 cm long, washed with water and dipped in a solution containing peptone, 10g; sucrose, 20g; K_2HPO_4, 0.1gm and $MgSO_4$, 0.25 gm per liter of water. These pieces were filled up to one-third capacity of 500ml conical flasks and were sterilized in an autoclave at 1.06kg/cm^2 for 20 minutes. The flasks were then inoculated separately with pure cultures of *the R. solani* grown on PDA plate and were incubated at 28±2ºC for colonization of *R. solani* and sclerotial formation. Transgenic plants, untransformed control plants along with a susceptible variety (IR 50) were inoculated with *R. solani* colonized typha pieces at maximum tillering stage. Typha pieces (8-10pieces/plant) were placed between the tillers, 5cm above the water level, ensuring that the tillers were tightly secured. The relative humidity in glass house was maintained between 80-90% and data were recorded one week after inoculation. Experiments

were conducted in four replications. Disease development was recorded and the disease index was calculated by integrating both the phases of disease development.

G. Disease Index (%)

Disease Index was calculated using infected tillers (%) and disease score (0-9 scale).

$$\text{Disease Index (\%)} = \frac{\text{Actual disease score}}{\text{Maximum score in the scale}} \times \text{Infected tillers (\%)}$$

III. RESULTS

A. Cloning of plant expression cassettes containing *Chi11* in binary vector of pSB11-*bar* of *Agrobacterium*

The 1.5kb DNA fragment consisting of rice endochitinase (*Chi11*) and its terminator sequences driven by CaMV 35 S promoter was excised with *Hind*III from the pAHG11 plasmid, and was cloned at the *Hind*III site of pSB11-*bar* intermediate vector. The resultant binary intermediate vector was named as pSB11-*bar*-Chi11 and was mobilized into *A. tumefaciens* strain LBA4404 by triparental mating and the resultant super-binary vectors were designated as pSB11-*bar*-*Chi11* (Fig. 1). Co-integration between pSB11*bar*-*Chi11* intermediate vector and pSB1 acceptor vector present in LBA4404 was verified through *Sal*I digestion. The pSB1 acceptor vector of LBA 4404, when digested with *Sal*I, showed a characteristic band of 5.3 kb. Whereas, the *Sal*I digested pSB111-*bar*-*Chi11* super-binary vectors revealed the absence of 5.3 kb band and presence of two additional bands of 3.1 kb and 7.3 kb (Data not shown). The LBA4404 strain, harboring super-binary vectors, was used for stable transformation of leading rice cultivars employing embryogenic calli derived from the immature embryos.

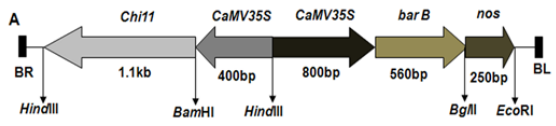

Fig 1. Restriction map of pSB111-*bar*-*Chil1*

B. Genetic transformation and production of transgenic rice plants

To insert *Chi11* gene into rice plants, embryogenic calli of rice (cv. Swarna) was co-cultivated with the *Agrobacterium* strain LBA4404 harbouring Ti-plasmid pSB111-*bar*-*Chi11*. A total number of 27 putative transformants were obtained from 1820 calli of Swarna. From these, 6 transformants were selected for further analyses based on their high tolerance to herbicide (0.25%) BASTA (Fig. 2).

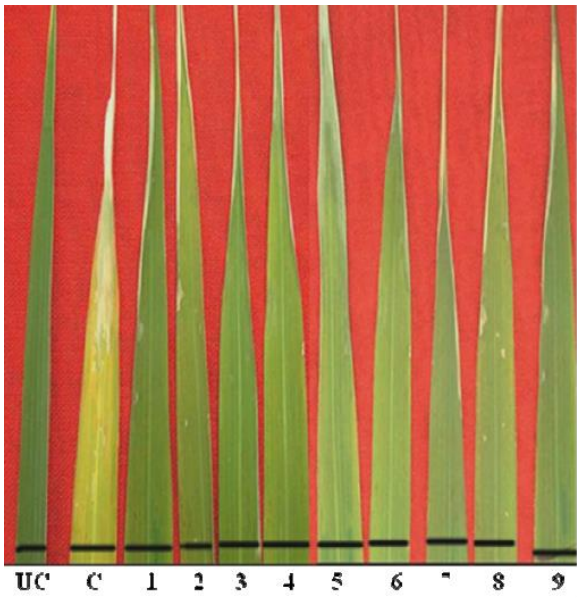

Fig 2. Basta treated leaves of putative *Chi11* transformants showing variable levels of tolerance to the herbicide. Lane. UC: Untransformed control plant without herbicide Basta treatment. Lane C: Untransformed control plant showing complete damage to the herbicide basta. Lanes 1-9: Different transformants of Chaitanya showing herbicide tolerance

C. Molecular analysis of primary (T_0) transgenic plants

Genomic DNA was isolated from the BASTA tolerant transgenic rice plants as well as from the untransformed control plants. PCR analysis of transgenic rice plants showed amplification of 750 bp and 600 bp products, representing *bar* and *Chi11* expression cassette sequences, while control plants failed to show such amplification (data not shown). Southern blot analysis was carried out using BASTA and PCR positive plants. When genomic DNA of

transgenic plants was digested with *Hind*III and probed with *Chi11* coding sequence, it showed hybridizable band of ~1.5 kb (Fig. 3A). Genomic DNA of different transgenic plants, digested with *Hind*III and probed with the *bar*, showed a distinct hybridizable band of >2.5 kb (Fig. 3B). These bands correspond to the expression units of *bar* and *Chi11* transgenes introduced into the transgenic rice plants.

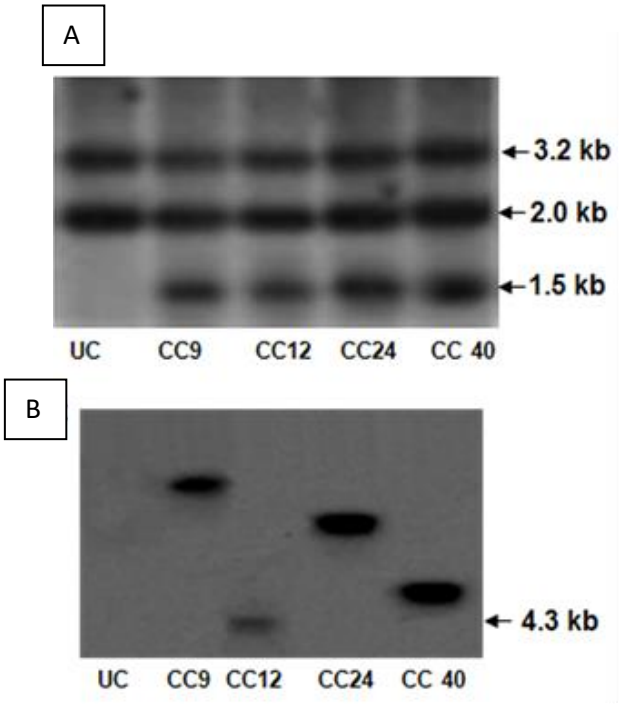

Fig 3. A: Genomic DNA digested with *Hind*III and probed with *Chi11*coding sequence. *Lane UC*: DNA from the untransformed control plant, *Lanes CC9, CC12, CC24* and *CC40* DNA from transgenic lines of Chaitanya. B: Genomic DNA digested with *Hind*III and probed with *bar* coding sequence. *Lane UC* DNA from the untransformed control plant, *Lanes CC9, CC12, CC24* and *CC40* DNA from transgenic lines of Chaitanya.

Conversely, the untransformed control plants failed to show any hybridizable band with both the probes.

Northern blot analysis was performed using the RNA from Southern positive plants to assess the expression of *Chi11*and *bar* genes in different transgenic rice lines; presence of a >980 bp hybridizable band of varied intensity was visualized in diverse transgenic lines (Fig. 4).

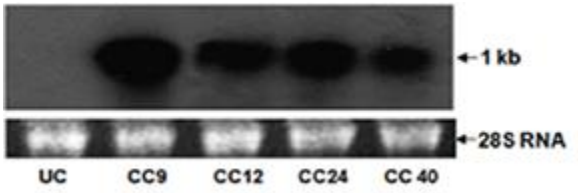

Fig 4. Northern blot analysis for the expression pattern of *Chi11* in transgenic rice lines. *Lane UC* RNA from untransformed control plant, *Lanes CC9, CC12, CC24* and *CC40* RNA from transgenic lines of Chaitanya. Ethidium bromide stained 28S rRNA band is shown under northern blot for amount of RNA loading.

Inheritance pattern of asal and bar genes in T_1 generation

To investigate the inheritance pattern of the transgenes, selfed seed collected from the primary (T_0) transformants were germinated and T_1 progenies were grown to maturity in the glass house. Six T_1lines of Swarna, viz., $SC_{9\text{-}1}$, $SC_{12\text{-}1}$, $SC_{14\text{-}1}$, $SC_{20\text{-}1}$, $SC_{24\text{-}1}$ and $SC_{40\text{-}1}$ were tested with the herbicide BASTA and were also subjected to fungal bioassays. In T_1 progenies, both the transgenes *bar* and *Chi11* showed a monogenic segregation of 3 resistant: 1 susceptible plant(s) besides co-segregation in a normal Mendelian fashion for BASTA tolerance as well as for insect resistance (Table. 1). These transgenic lines were healthy and were found similar to that of untransformed control plants for various morphological characters with normal seed fertility.

Table 1. Inheritance pattern of *bar-Chi11* transgenes, and fungal bioassays for LB and SB in T1 generation.

T_1 Progenies	PPT test/ Bioassay	Total no. of plants tested	No. of Tolerant/ resistant plants	No. of susceptible plants	Expected ratio	χ 2 value
CN control	PPT	28	0	28	-	-
CC_{9-1}	PPT	32	23	9	3:1	0.1666
CC_{12-1}	PPT	34	24	10	3:1	0.3529
CC_{24-1}	PPT	26	20	6	3:1	0.0512
CC_{40-1}	PPT	34	25	9	3:1	0.0390
CN control	LB	24	0	24	-	-
CC_{9-1}	LB	25	18	7	3:1	0.1200
CC_{12-1}	LB	26	17	9	3:1	1.2820
CC_{24-1}	LB	36	29	7	3:1	0.5925
CC_{40-1}	LB	32	25	7	3:1	0.1666
CN control	SB	35	0	35	-	-
CC_{9-1}	SB	34	23	11	3:1	0.9803
CC_{12-1}	SB	29	23	6	3:1	0.2873
CC_{24-1}	SB	28	25	3	3:1	3.0476
CC_{40-1}	SB	34	23	11	3:1	0.9803

CN control: Untransformed Chaitanya control; CC9-1, CC12-1, CC24-1 and CC40-1 *Chi11* transgenic rice lines; PPT: phosphinothricin; LB: leaf blast; SB: Sheath blight. The inheritance pattern of transgenes showed 3:1 ratio and the calculated $\chi 2$ values were significant at p value < 0.05.

Impact of *Chi11* on fungal pathogens

Comprehensive *in planta* bioassay experiments were performed to test the antifungal activity of the *Chi11* gene, on T_1 and T_2 (homozygous) transgenic lines, for two major fungal pathogens of rice. The performance of transgenic lines expressing *Chi11*gene, untransformed controls, along with susceptible (HR12) and resistant (IR 64) checks, were evaluated against the blast fungus *Magnaporthe grisea*. The response to disease was measured on the basis of SES scoring. The score values represent the mean of two replicates (~40 plants), from three independent experiments, conducted in nursery beds. Blast disease index was measured based on the number of infected lesions per leaf, lesion size and percentage affected leaf area, involving five individual plants from each experiment. The nature and frequency of lesions on leaves of representative plants are shown in **Fig. 5**. The susceptible check HR12, exhibited larger necrotic lesions, with >85% diseased leaf area, while untransformed Swarna showed diseased leaf area of 79%. *Chi11*-Swarna transformants disclosed reduction in mean number of lesions per leaf, mean size of lesions and mean diseased area as compared to the untransformed controls. In SC_{9-1-12}, SC_{12-1-9}, $SC_{24-1-13}$ and SC_{40-1-5} *Chi11*-transformants a mean number of 3.60±0.54, 6.00±0.70, 6.00±0.70 and 7.20±0.83 lesions per leaf were observed compared to 9.80±0.83 lesions per leaf in untransformed Swarna. Also, the four transformants exhibited smaller lesions of 2.44±0.34, 2.56±0.11, 2.68±0.19 and 3.30±0.24 mm, compared to 4.18±0.19 mm lesions observed in control plants. Compared to untransformed control, *Chi11*-transformants of Swarna showed 43 to 79% reduction in the disease index. Transgenic lines expressing *Chi11* exhibited a SES score of 3 to 5, compared to SES scores 1 to 3 of resistant (IR 64) and 7 to 9 of susceptible (HR12) checks.

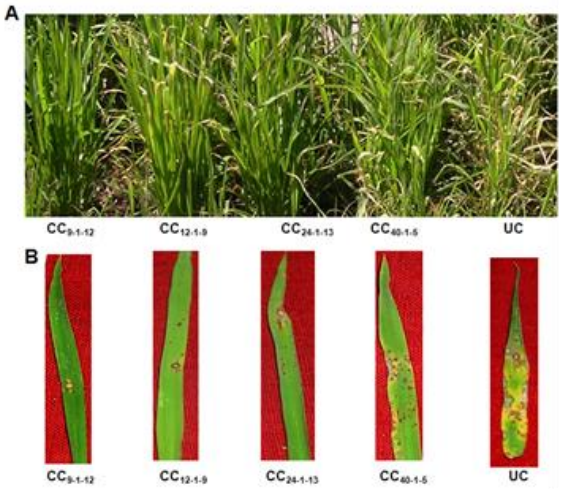

Fig 5. Evaluation of *Chi11*-transgenic lines against rice leaf blast disease. A Seedling beds of *Chi11*-transgenic lines of Chaitanya along with untransformed controls exhibiting disease symptoms after 15 days of infection with *M. grisea*. B Single leaf from *Chi11*-transgenic lines of Chaitanya along with untransformed controls showing disease symptoms after 15 days of infestation. $CC_{9\text{-}1\text{-}12}$, $CC_{12\text{-}1\text{-}9}$, $CC_{24\text{-}1\text{-}13}$ and $CC_{40\text{-}1\text{-}5}$ Chaitanya *Chi11*-transgenic rice lines, *UC* Chaitanya untransformed control plants.

Transgenic rice plants expressing *Chi11* gene, untransformed controls along with susceptible (IR50) and resistant (Swarnadhan) checks, were evaluated against the sheath blight disease caused by the fungus *Rhizoctonia solani*. Distinct lesions were developed within 4 to 5 days after inoculation on both untransformed controls and transgenic plants. However, the size of lesions was strikingly smaller in transgenic plants compared to controls even 10 days after inoculation with the pathogen (Fig. 6). Homozygous *Chi11*-transformants of Swarna with variable expression were showed reduced disease symptoms (Fig. 6). Among four Swarna *Chi11*-transformants ($SC_{9\text{-}1\text{-}12}$, $SC_{12\text{-}1\text{-}9}$, $SC_{24\text{-}1\text{-}13}$ and $SC_{40\text{-}1\text{-}5}$), a mean number of 2.0±0.00, 3.6±0.89, 4.6±0.54 and 4.8±0.44 tillers per plant were found infected compared to 8.8±1.64 in the untransformed control. Similarly, all the four transformants exhibited marked decreases in vertical disease spread. In four Swarna transgenics, upper most lesions were observed at mean plant heights of 22.2±1.30, 30.6±3.97, 33.0±1.00 and 43.0±1.58cm compared with 54.0±5.78 cm in the untransformed control (Fig. 6; Suppleentary file; Table 6). *Chi11* transformants disclosed a mean disease reduction of 54 to 91% when compared to the untransformed control plants (Fig. 6; Suppleentary file; Table 6). Transgenic lines expressing *Chi11* showed a SES score of 3 to 5, while the scores of resistant (Swarnadhan) and susceptible (IR50) checks varied from 3 to 5 and 7 to 9, respectively.

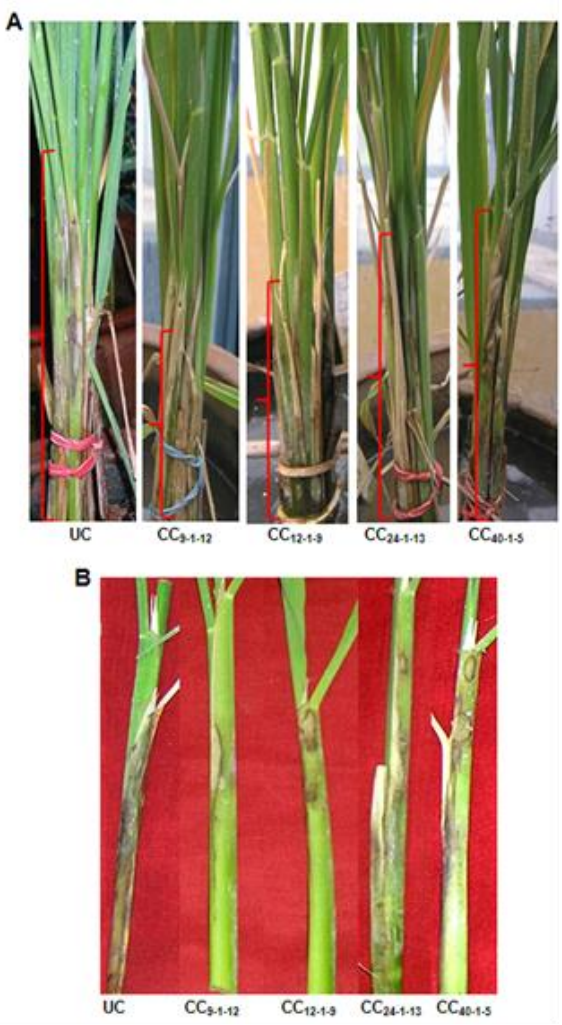

Fig 6. Evaluation of *Chi11*-transgenic lines against sheath blight disease. A *Chi11*-transgenic lines of Chaitanya along with untransformed controls exhibiting symptoms after 20 days of infection with *R.solani* at maximum tillerring stage. {- Indicates vertical disease spread of the disease symptoms. B Single tiller from *Chi11*-transgenic lines of Chaitanya along with untransformed controls showing disease symptoms after 20 days of infestation. *UC* Chaitanya untransformed control plants, $CC_{9\text{-}1\text{-}12}$, $CC_{12\text{-}1\text{-}9}$, $CC_{24\text{-}1\text{-}13}$ and $CC_{40\text{-}1\text{-}5}$ Chaitanya *Chi11*-transgenic rice lines.

Characterization of *Chi11*-transgenic lines for endochitinase activity

Endochitinase activity assay was performed with total soluble leaf protein extracts of four Swarna (T_2) homozygous *Chi11*-transformants ($SC_{9\text{-}1\text{-}12}$, $SC_{12\text{-}1\text{-}9}$,

$SC_{24\text{-}1\text{-}13}$ and $SC_{40\text{-}1\text{-}5}$) along with the untransformed control plants, using the substrate 4-methylumbelliferyl-β-D-*N*, *N´*, *N´´*-triacetylchitotrioside. Mean specific activity of endochitinase in the control, $SC_{9\text{-}1\text{-}12}$, $SC_{12\text{-}1\text{-}9}$, $SC_{24\text{-}1\text{-}13}$ and $SC_{40\text{-}1\text{-}5}$ transformants, were 1.28±0.05, 15.13±0.18, 9.99±0.05, 7.36±0.09 and 4.83±0.18 nanomoles product /min/mg protein, respectively (Fig 7). The transgenic plants exhibited 3.54 - to 13.85 - fold higher specific activity of endochitinase than that of untransformed control plants.

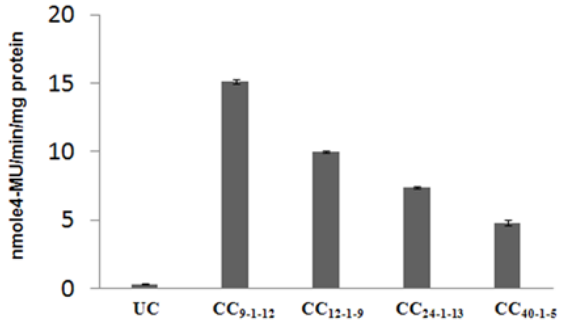

Fig 7. Endochitinase activity in homozygous *Chi11* transformants and untransformed control. The endochitinase activity is expressed as nmole 4-MU/min/mg protein. Each column represents the mean endochitinase activity from three plants of each transgenic line. *UC* Chaitanya untransformed control plants, *CC9-1-12*, *CC12-1-9*, *CC24-1-13* and *CC40-1-5* Chaitanya *Chi11*-transgenic rice lines. Bars indicate mean ± Standard Error (S.E), Differences between the mean values of untransformed control vs. transgenic lines were significant at p value <0.0001 (unpaired t-test).

Identification of homozygous transgenic lines using PPT sensitivity test in T_2 generation

Homozygous transgenic rice lines, expressing *BjNPR1* / *Chi11*genes, were identified in T_2 generation, employing PPT sensitivity test, using selfed seed collected from T_1 plants. Selfed seed of 32 T_2 progenies derived from $SMN_{2\text{-}1}$ and $SMN_{5\text{-}1}$ T_1 plants, when germinated on MS medium containing PPT, showed 6 T_2 progenies with complete germination, 18 progenies segregated into 3:1 ratio, and the seeds of 8 progenies failed to show germination (**Table 2**). Similarly, selfed seed of 48 T_2 progenies, obtained from $CN_{2\text{-}1}$, $CN_{12\text{-}1}$ and $CN_{76\text{-}1}$ T_1 plants, after germination on PPT containing MS medium, showed 10 T_2 progenies with complete germination, 27 progenies showed segregation in a 3:1 ratio, while 11progenies failed to germinate (**Table 2**). Likewise, selfed seed of 64 T_2 progenies obtained from $SC_{9\text{-}1}$, $SC_{12\text{-}1}$, $SC_{24\text{-}1}$ and $SC_{40\text{-}1}$ T_1 plants, when subjected to PPT sensitivity test, 12 T_2 progenies, showed complete germination on MS medium supplemented with 5 mg/l PPT, 38 progenies disclosed segregation in a 3:1 ratio, while 14 T_2 progenies failed to show germination (**Table 2**). Based on progenies showing 100% germination on PPT medium, two homozygous lines of *BjNPR1*-SM ($SMN_{2\text{-}1\text{-}15}$ and $SMN_{5\text{-}1\text{-}10}$), three homozygous lines of Chaitanya ($CN_{2\text{-}1\text{-}4}$, $CN_{12\text{-}1\text{-}10}$ and $CN_{76\text{-}1\text{-}5}$) and four homozygous lines of *Chi11*-Swarna ($SC_{9\text{-}1\text{-}12}$, $SC_{12\text{-}1\text{-}9}$, $SC_{24\text{-}1\text{-}13}$ and $SC_{40\text{-}1\text{-}5}$) transformants were selected for further studies. Moreover, homozygosity of selected *BjNPR1*- and *Chi11*-transgenic lines were validated through Basta test. All the plants of selected homozygous lines invariably exhibited tolerance to 0.25% Basta, and were employed for fungal and bacterial bioassays.

Table 2. Identification of *bar-Chi11* homozygous lines in T_2 progenies of Swarna *Chi11* transformants using PPT sensitivity test

T_2 Progenies	No. of seeds tested	No. of germinated seeds	No. of ungerminated seeds	Expected ratio	$\chi 2$ value	p-value

$SC_{9\text{-}1}$ plant Progenies						
$SC_{9\text{-}1\text{-}1}$	26	21	5	3:1	0.461	0.496
$SC_{9\text{-}1\text{-}2}$	27	21	4	3:1	1.493	0.221
$SC_{9\text{-}1\text{-}3}$	29	23	8	3:1	0.103	0.747
$SC_{9\text{-}1\text{-}4}$	27	21	10	3:1	2.086	0.148
$SC_{9\text{-}1\text{-}5}$	26	17	10	3:1	2.512	0.112
$SC_{9\text{-}1\text{-}6}$	29	23	6	3:1	0.287	0.591
$SC_{9\text{-}1\text{-}7}$	28	25	3	3:1	3.047	0.080
$SC_{9\text{-}1\text{-}8}$	26	22	4	3:1	1.282	0.257
$SC_{9\text{-}1\text{-}9}$**	24	24	0	-	-	-
$SC_{9\text{-}1\text{-}10}$	23	0	23	-	-	-
$SC_{9\text{-}1\text{-}11}$	28	0	28	-	-	-
$SC_{9\text{-}1\text{-}12}$**	27	27	0	-	-	-
$SC_{9\text{-}1\text{-}13}$	29	0	29	-	-	-
$SC_{9\text{-}1\text{-}14}$	26	21	5	3:1	0.461	0.496
$SC_{9\text{-}1\text{-}15}$	26	0	26	-	-	-
$SC_{9\text{-}1\text{-}16}$**	26	26	0	-	-	-
$SC_{12\text{-}1}$ plant progenies						
$SC_{12\text{-}1\text{-}1}$	25	20	5	3:1	0.039	0.843
$SC_{12\text{-}1\text{-}2}$	26	20	6	3:1	0.461	0.496
$SC_{12\text{-}1\text{-}3}$	26	21	5	3:1	0.461	0.496
$SC_{12\text{-}1\text{-}4}$**	24	24	0	-	-	-
$SC_{12\text{-}1\text{-}5}$	27	23	4	3:1	1.493	0.221
$SC_{12\text{-}1\text{-}6}$	27	0	27	-	-	-
$SC_{12\text{-}1\text{-}7}$	26	16	10	3:1	2.512	0.112
$SC_{12\text{-}1\text{-}8}$	22	0	22	-	-	-
$SC_{12\text{-}1\text{-}9}$**	25	25	0	-	-	-
$SC_{12\text{-}1\text{-}10}$	24	21	3	3:1	2.000	0.157
$SC_{12\text{-}1\text{-}11}$	28	21	7	3:1	0	0
$SC_{12\text{-}1\text{-}12}$	25	0	25	-	-	-
$SC_{12\text{-}1\text{-}13}$**	29	29	0	-	-	-
$SC_{12\text{-}1\text{-}14}$	32	23	9	3:1	0.166	0.683
$SC_{12\text{-}1\text{-}15}$	34	25	9	3:1	0.039	0.843
$SC_{12\text{-}1\text{-}16}$	36	24	12	3:1	1.333	0.248

Table 2 (Continued);

T_2Progenies	No. of seeds tested	No. of germinated seeds	No. of ungerminated seeds	Expected ratio	$\chi 2$ value	p-value
$SC_{24\text{-}1}$ plant Progenies						
$SC_{24\text{-}1\text{-}1}$	28	0	28	-	-	-
$SC_{24\text{-}1\text{-}2}$	32	23	9	3:1	0.1666	0.6831
$SC_{24\text{-}1\text{-}3}$**	28	28	0	-	-	-
$SC_{24\text{-}1\text{-}4}$	26	20	6	3:1	0.0512	0.8208
$SC_{24\text{-}1\text{-}5}$	34	25	9	3:1	0.0390	0.8430
$SC_{24\text{-}1\text{-}6}$	24	0	24	-	-	-
$SC_{24\text{-}1\text{-}7}$	25	18	7	3:1	0.1200	0.7290
$SC_{24\text{-}1\text{-}8}$**	26	26	0	-	-	-
$SC_{24\text{-}1\text{-}9}$	36	29	7	3:1	1.2820	0.4414
$SC_{24\text{-}1\text{-}10}$	32	25	7	3:1	0.5925	0.6831
$SC_{24\text{-}1\text{-}11}$	35	0	35	-	0.1666	-
$SC_{24\text{-}1\text{-}12}$	34	23	11	3:1	-	0.3221
$SC_{24\text{-}1\text{-}13}$**	26	26	0	-	0.9803	-
$SC_{24\text{-}1\text{-}14}$	29	23	6	3:1	0.2873	0.5919
$SC_{24\text{-}1\text{-}15}$	28	25	3	3:1	3.0476	0.0809
$SC_{24\text{-}1\text{-}16}$	24	0	24	-	-	-
$SC_{40\text{-}1}$ plant progenies						
$SC_{40\text{-}1\text{-}1}$	32	23	9	3:1	0.166	0.6831
$SC_{40\text{-}1\text{-}2}$	34	24	10	3:1	0.352	0.5525
$SC_{40\text{-}1\text{-}3}$	26	0	26	-	-	-
$SC_{40\text{-}1\text{-}4}$	34	25	9	3:1	0.039	0.843
$SC_{40\text{-}1\text{-}5}$**	24	24	0	-	-	-
$SC_{40\text{-}1\text{-}6}$	25	18	7	3:1	0.120	0.729
$SC_{40\text{-}1\text{-}7}$	26	17	9	3:1	1.282	0.257
$SC_{40\text{-}1\text{-}8}$	24	16	8	3:1	0.888	0.3458
$SC_{40\text{-}1\text{-}9}$**	28	28	0	-	-	-
$SC_{40\text{-}1\text{-}10}$	35	24	11	3:1	0.771	0.3798
$SC_{40\text{-}1\text{-}11}$	34	23	11	3:1	0.980	0.3221
$SC_{40\text{-}1\text{-}12}$	29	0	29	-	-	-
$SC_{40\text{-}1\text{-}13}$	28	25	3	3:1	3.047	0.0809
$SC_{40\text{-}1\text{-}14}$**	29	29	0	-	-	-
$SC_{40\text{-}1\text{-}15}$	36	29	7	3:1	0.592	0.4414
$SC_{40\text{-}1\text{-}16}$	24	0	24	-	-	-

** = T_2 Homozygous lines $\chi 2$ calculated < $\chi 2$ tabulated 3.841 Significant at $P<0.05$

IV. DISCUSSION

Development of durable resistance in crop plants to a wide range of pathogens often involves huge resources as well as efforts when traditional breeding methods are adopted. In recent times, genetic engineering of plants for disease resistance has become an attractive approach in terms of cost and efficacy [64]. Disease resistance of crops against pathogens depends primarily on the timely recognition of pathogens apart from instant activation of effective defense response in plants. Effectiveness of *AtNPR1* and rice *Chi11* genes in conferring increased non-specific disease-resistance was demonstrated in other crop plants including tobacco [44], Melon [45], pea nut [46], canola [47],

Maize [48], straw berry [49], cucumber [50], tobacco and tomato [51]. Exploitation of systemic acquired resistance (SAR) for engineering crops for disease-resistance serves as a potent, broad based mechanism against an array of pathogens [65]. This investigation deals with the effectiveness of SAR-related genes for enhanced resistance to multiple pathogens by over-expressing *B. juncea* non-expressor of pathogenesis-related gene 1 (*BjNPR1*) and *O. sativa* endochitinase gene (*Chi11*) in indica rice. *A. tumefaciens* (LBA4404), harbouring super-binary vectors containing *BjNPR1* and *Chi11*, were employed as candidate genes for development of transgenic rice plants.

Ti-plasmid intermediate vector, pSB11-*bar*-*BjNPR1*, was constructed by cloning the 1.74kb cDNA coding region (AY667498) of *BjNPR1* at *Bam*HI site of pSB11-*bar*. *Bam*HI digestion of the recombinant plasmid DNA released 1.74kb *BjNPR1* coding sequence and 8.7kb of pSB11-*bar* (Fig. 2), indicating the presence of *BjNPR1* coding region. The orientation of cloned *BjNPR1* in intermediate vector was analyzed by *Xho*I digestion, which resulted in three fragments of 168bp, 191bp and ~10kb (Fig. 4), thus revealing the correct orientation of *BjNPR1* gene downstream to CaMV35S promoter. The Ti-plasmid pSB11-*bar*-*Chi11* was constructed by cloning the 1.5kb *Chi11* expression unit at *Hind*III site of pSB11-*bar* (Fig. 3). *Hind*III digestion of the recombinant plasmid DNA released ~8kb fragment of pSB11-*bar* along with 1.5kb fragment, implying the presence of *Chi11* gene expression cassette. Also, the *Eco*RI and *Bam*HI digestion of pSB11-*bar*-*Chi11* plasmid produced two fragments of 2.1kb and 7.4kb (Fig. 4). The release of 2.1kb fragment suggests that the two expression units of *Chi11* and *bar* in the T-DNA are in opposite orientation (Figs.5 & 6).

After triparental mating, recombinant clones selected on YEP medium supplemented with 50mg/l spectinomycin and 10mg/l tetracycline were subjected to molecular analyses. The acceptor vector (pSB1) DNA was digested with *Sal*I released the 5.3kb fragment corresponding to the homologous region between acceptor and intermediate vectors. *Sal*I digestion of pSB111-*bar*-*BjNPR1* (Fig. 7A) and pSB111-*bar*-*Chi11* (Fig. 8A) plasmids, released two specific fragments of 3.1kb and 7.3kb. Further, the absence of 5.3kb fragment pertaining to homologous region in pSB111-*bar*-*BjNPR1* and pSB111-*bar*-*Chi11* vectors confirm the successful recombination leading to formation of the co-integrate super-binary vector with *bar* and *BjNPR1* / *Chi11* expression cassettes. The results of *Sal*I digestion amply demonstrate the formation of co-integrate super-binary vector with intact expression cassettes of *bar*, *BjNPR1* and *Chi11*genes.

To introduce *BjNPR1* gene for developing disease resistant transgenic rice, embryogenic calli of Samba Mahsuri (SM) and Chaitanya — found susceptible to various fungal and bacterial diseases — were co-cultivated with the LBA4404 harbouring pSB111-*bar*-*BjNPR1* vector. Similarly, embryogenic calli of Swarna was co-cultivated with the *Agrobacterium* harbouring pSB111-*bar*-*Chi11* vector. A total number of 9 SM and 34 Chaitanya putative *BjNPR1*-transformants, and 27 Swarna *Chi11*-transformants were produced (Table 5). SM, Chaitanya and Swarna showed differential transformation frequency of 0.38%, 1.71% and 1.48%, respectively, owing to differences in the genetic milieu of rice varieties. The putative transformants showed varied levels of tolerance to herbicide Basta, presumably, because of the differential expression of the *bar* gene.

BjNPR1-SM and Chaitanya, and *Chi11*-Swarna transformants, which showed consistently higher tolerance to Basta in repeated tests, were further subjected to molecular analyses for confirmation of integration and expression of transgenes. PCR analyses of Basta tolerant *BjNPR1*-transformants revealed the amplification of 750bp and 600bp products corresponding to *bar* and *BjNPR1* transgenes indicating the presence of these genes in the genome of transformants. (Figs. 15A & 15B). Southern blots of *Bam*HI digested genomic DNA of *BjNPR1* transformants, when probed with the radiolabelled *BjNPR1*, revealed a specific hybridizable band of ~1.8kb in each of the transformants, suggesting the stable integration of *BjNPR1* in different primary transformants of rice (Fig. 16A). Appearance of different hybridizable bands of >2.6kb corresponding to the *bar* expression cassette with *bar* coding sequence in the *Bam*HI digested genomic DNA of these plants, indicates the single copy and independent nature of the transformants (Fig. 16B). Single copy

integration of transgene(s) is essential to achieve predictable patterns of inheritance and to eliminate the problem of gene silencing in transgenic plants [66].

Molecular analyses of Basta tolerant *Chi11*-transformants revealed stable integrations of *bar* and *Chi11* transgenes in their genomes (Figs. 17A, 17B, 18A &18B). Appearance of different hybridizable bands of >2.5kb corresponding to the *bar* expression cassette, denotes the independent nature of transgene integration in different transformants when *bar* coding sequence was used as a probe (Fig. 18B). *Hind*III digested genomic DNA of these plants, when probed with the radiolabelled *Chi11*, disclosed a specific hybridizable band of ~1.5kb (Fig. 18A) in different transformants, specifying *Chi11* transgene integration in primary transformants of rice. Apparently, the common bands observed in control and transgenic plants represent the endogenous chitinase (*chi*) genes of rice [67]. The specific bands observed on Southern blots of transgenic plants confirm the presence of *bar* and *BjNPR1* / *Chi11* transgenes. Conversely, the untransformed control plants failed to show specific hybridizable bands with these probes. Northern blot analyses showed variable expression of *bar*, *BjNPR1* and *Chi11* genes in the primary transgenic plants as evidenced by varied intensity of the hybridizable bands of >600bp, >1.8kb and >1.0kb when *bar*, *BjNPR1* and *Chi11* genes were employed as probes (Figs. 19-22). The level of transgene expression was variable and influenced by many factors, such as, integration site, transgene copy number, transgene locus configuration, epigenetic silencing factors, and flanking matrix attachment regions. It is well established that the transgenes present in multiple copies as well as transgenes that are related to homologous endogenous sequences invariably show gene inactivation. *Indica* rice varieties are known to be recalcitrant to *Agrobacterium*-mediated genetic transformation. *Agrobacterium*-mediated transformation, compared to other techniques, proved superior by virtue of its higher transformation efficiency in conjunction with predominant single copy transgene integration. High frequency of gene transfer was reported in *indica* rice by employing super-binary vectors of Agrobacterium [68], [69].

To establish the definitive transgenic nature of primary transformants, the inheritance pattern of transgenes was analysed in the T_1 and T_2 generations. Segregation analyses of transgenes in T_1 progenies revealed a monogenic ratio of 3 resistant: 1 susceptible plant(s) for herbicide tolerance as well as fungal and bacterial resistance (Figs. 23 & 24; Tables 6 & 7), suggesting that these genes are stably integrated into the rice genome. The co-segregation of transgenes affirms that both *bar*-*BjNPR1* and *bar*-*Chi11* are integrated and manifest as a single locus.

Homozygous transgenic rice lines, expressing *BjNPR1* / *Chi11*genes, were identified in T_2 generation, employing PPT sensitivity test. Selfed seed of 144 T_2 progenies derived from *BjNPR1*-SM and Chaitanya, and *Chi11*-Swarna T_1 plants, when subjected to PPT sensitivity test, 28 T_2 progenies showed complete germination on MS medium supplemented with 5 mg/l PPT, 83 T_2 progenies disclosed a 3:1 ratio, while 33 T_2 progenies failed to show seed germination (Figs. 25 & 26; Tables 8 & 9), owing to segregation of transgenes in a monogenic ratio of 1homozygous: 2 hemizygous: 1 azygous plants. Based on PPT sensitivity test, two homozygous lines of *BjNPR1*-SM ($SMN_{2\text{-}1\text{-}15}$ and $SMN_{5\text{-}1\text{-}10}$), three homozygous lines of Chaitanya ($CN_{2\text{-}1\text{-}4}$, $CN_{12\text{-}1\text{-}10}$ and $CN_{76\text{-}1\text{-}5}$) and four homozygous lines of *Chi11*-Swarna ($SC_{9\text{-}1\text{-}12}$, $SC_{12\text{-}1\text{-}9}$, $SC_{24\text{-}1\text{-}13}$ and $SC_{40\text{-}1\text{-}5}$) transformants were selected for further evaluations. All the plants of selected homozygous lines invariably exhibited uniform tolerance to 0.25% Basta, demonstrating their homozygous nature.

In planta fungal and bacterial bioassays amply indicate that the expression of *BjNPR1* gene in transgenic rice lines imparts substantial resistance against leaf blast, sheath blight and bacterial leaf blight diseases, as evidenced by decreased disease index (Tables 10,12 & 14). The T_2 progenies of five *BjNPR1* homozygous transgenic lines, when subjected to fungal and bacterial bioassays, exhibited high-level (3-5 score on a 0-9 scale) resistance, testifying that *BjNPR1* affords broad-spectrum resistance to bacterial and fungal diseases. *BjNPRI*-Chaitanya and SM transformants when evaluated against rice leaf blast, disclosed reduction in mean number of lesions per leaf, mean size of lesions and mean diseased area as compared to the untransformed controls (Figs. 28A & 28B). In *BjNPR1* transformants, a mean number of 3.20±0.83 to 5.80±0.44 lesions per leaf were observed compared to 9.20±0.83 and 8.80±0.83 lesions in SM

and Chaitanya controls (Table 10). Mean lesion size ranging from 2.18±0.10 to 3.18±0.14 mm were noticed on the leaves of *BjNPR1*-transformants, compared with 4.28±0.16 and 4.26±0.18 mm lesions observed in untransformed controls (Fig. 29; Table 10). *BjNPR1* transformants exhibited 34 to 65% reduction in mean number of lesions per leaf and 25 to 49% reduction in mean size of lesions per leaf. When compared to untransformed controls, *BjNPR1* transformants of SM and Chaitanya showed disease reduction of 50 to 79% (Fig. 30).

Furthermore, *BjNPR1* transgenic rice lines were evaluated against sheath blight disease caused by *Rhizoctonia solani*. Distinct lesions were developed within 4 to 5 days after inoculation on both untransformed controls and transgenic plants. However, the lesions were strikingly smaller in transgenic plants compared to the controls even 10 days after inoculation with the pathogen (Figs. 35A & 35B). SM and Chaitanya transformants expressing *BjNPR1* transgene revealed significant reduction in the mean number of infected tillers per plant, relative plant height of the uppermost lesion and lesion size compared to untransformed controls (Fig. 36; Table 12). A mean number of 2.20±0.44 to 4.40±0.89 infected tillers per plant were observed in *BjNPR1* transformants compared to 9.20±1.48 and 7.80±0.44 infected tillers per plant in untransformed controls. In transgenics, upper most lesions were observed at mean plant heights of 21.80±0.44 to 31.60±0.89 cm in *BjNPR1* transformants compared with 46.02±0.74 and 44.80±2.04 cm in the untransformed SM and Chaitanya controls (Fig. 36; Table 12). Overall, *BjNPR1* transformants exhibited 43 to 76% reduction in mean number of infected tillers per plant and 29 to 52% reduction in the vertical disease spread of plant. Transgenic lines expressing *BjNPR1* disclosed a mean disease reduction of 65 to 91% as compared to the untransformed controls (Fig. 37; Table 12).

BjNPR1 transgenic rice lines also showed significant levels of resistance towards BLB disease. Untransformed SM and Chaitanya plants developed lesions of 15.16±0.75 and 14.16±0.75 cm in a total leaf length of 15.66±0.51and 15.16±0.75 cm, respectively; whereas *BjNPR1* transformants exhibited a lesion length of 3.33±0.51 to 5.50±0.54 cm (Table 14). These transformants revealed a lesion length ranging from 20 to 40% compared to 90 to 95% lesion length observed in the untransformed controls (Fig. 42; Table 14). Leaves of *BjNPR1*-transgenic lines showed distinctly smaller lesions as compared to untransformed controls. In different transgenic lines, the lesion area was substantially reduced by 60 and 76 % compared to their respective untransformed controls (Fig. 43; Table 14).

The overall results clearly suggest that *BjNPR1* expressing transgenic rice lines exhibit higher-levels of resistance against diseases caused by three pathogens. It was reported that over-expression of *AtNPR1* in *Arabidopsis*, rice, tomato and wheat results in increased resistance to various fungal and bacterial pathogens. Similarly, over-expression of *OsNH1* in rice promoted enhanced resistance to the bacterial blight. Furthermore, ectopic expression of *AtNPR1* and *OsNH1* genes in rice was found associated with the constitutive expression of certain rice defense genes. Most of the rice cultivars were found to show higher base-levels of than that observed in *Arabidopsis*, maize and wheat plants. Increased levels of salicylates were shown to promote the constitutive expression of *NPR1*-regulated pathways, thereby conferring broad-spectrum resistance to pathogens in transgenic rice over-expressing *AtNPR1* or *OsNH1* genes [70]-[72].

NPR1 is a critical component of the salicylic acid (SA)-mediated signal transduction pathway leading to the induction of various defense genes, and it is known to act downstream to SA in the SAR pathway. *NPR1* is generally expressed at low levels in healthy uninfected plants and functions as a transcription co-activator of genes involved in the resistance signalling pathways. However, the expression of *NPR1* is enhanced two- to three- folds upon pathogen infection or treatment with SA or its functional analogs. Although the interaction of NPR1 with TGA factors is not constitutive, yet it was found to be regulated during the establishment of SAR. The redox regulation of TGA1 represents a level of control for the interaction between NPR1 and TGA transcription factors during the SAR establishment [74]. Different TGA factors probably play an indirect role in regulating PR gene expression by interacting with NPR1 to upregulate the expression of other DNA binding transcription factors that are rate limiting to PR gene expression. These

TGA factors also bind to W-box *cis* elements present in the promoters of genes that encode PR proteins [74], as well as genes belonging to certain WRKY transcription factors. Investigations employing SA analogs and pathogens revealed the existence of SAR-like pathways in rice. Treatment of rice seedlings with BTH induced the accumulation of defense gene transcripts, resulting in resistance to the fungal pathogen *M. grisea* [75]. The accrued results of the present study — with transgenic rice over-expressing BjNPR1 affording enhanced resistance to both fungal and bacterial pathogens — amply indicate a definitive role for BjNPR1 in SAR pathway. Although the precise role of SA is unclear, we envisage the functional involvement of BjNPR1 in the activation of downstream components of the SAR pathway leading to broad-spectrum resistance against different pathogens in rice.

In planta fungal bioassays on Swarna transformants, over-expressing *Chi11* gene, conveyed significant resistance against sheath blight and leaf blast diseases, as evidenced by decreased disease index (Tables 11&13). The T_2 progenies of four *Chi11* homozygous transgenic lines, when subjected to fungal bioassays, exhibited high-level (1-5 score on a 0-9 scale) resistance, testifying that *Chi11* confers resistance to both the fungal pathogens. *Chi11*- Swarna transformants, when evaluated against rice leaf blast, revealed explicit reduction in mean number of lesions per leaf, mean size of lesions and mean diseased area as compared to the untransformed controls (Fig. 31). In *Chi11*-Swarna lines, the mean number of lesions ranged between 3.60±0.54 and 7.20±0.83 per leaf compared to 9.80±0.83 lesions in untransformed Swarna plants. Also, the four transformants exhibited smaller lesions of 2.44±0.34 to 3.30±0.24 mm as compared to 4.18±0.19 mm lesions observed in control plants (Figs. 32 & 33; Table 11). Further, *Chi11* transformants disclosed 26 to 63% reduction in mean number of lesions per leaf and 21 to 42% reduction in the mean size of lesions when compared to control plants. *Chi11*- Swarna lines also showed 43 to 79% reduction in the disease index compared to untransformed control plants (Fig. 34; Table 11)

Li et al [76] reported that over-expression of rice chitinases *Cht-2* / *Cht-3* in transgenic rice lines conferred enhanced resistance to *M. grisea* in a non-race-specific manner. Transgenic rice lines expressing *McCHIT1* exhibited greater resistance to *M. grisea* with a reduction of 30.0 to 85.7% in disease index compared to that of control plants [77]. Moreover, the resistance conferred by *Cht-2* / *Cht-3* and *McCHIT1* genes was not race specific to rice blast fungus. As such, these genes might bestow nonspecific, durable resistance against new races of blast fungus, besides increased resistance against other fungal diseases [77].

Chi11-transformants of Swarna with variable expression were also subjected to sheath blight bioassays. Among four Swarna *Chi11*- transgenic lines, a mean number of 2.0±0.10 to 4.8±0.44 tillers per plant were found infected as compared to 8.8±1.64 infected tillers observed in the untransformed control plants. In Swarna transgenic lines, upper-most lesions were observed at a mean plant height of 22.2±1.30 to 43.0±1.58cm compared with 54.0±5.78 cm in the control plants (Fig. 39; Table 13). Overall, *Chi11* transformants exhibited 45 to 77% reduction in mean number of infected tillers per plant and 20 to 58% reduction in the vertical-disease-spread of plants. Also, *Chi11* lines disclosed a mean disease reduction of 54 to 90% when compared to untransformed control plants (Fig. 40; Table 13).

Endochitinase activity assay was performed using total soluble leaf protein extracts of *Chi11*-transformants and that of untransformed control plants. Mean specific activity of endochitinase in different *Chi11*-Swarna transformants ranged between 4.83±0.18 and 15.13±0.18 nanomoles product/min/mg protein (Table 15). The transgenic plants disclosed 3.54- to 13.85- fold higher specific activity of chitinase in comparison with the untransformed control plants (Fig. 44). All the *Chi11* transgenic plants expressing high levels of endochitinase were found healthy and were similar to that of untransformed plants for various morphological characters with normal seed fertility. Bolar *et al*. [78] reported that apple transformants expressing higher levels of *T. harzianum* endochitinase gene showed reduction in plant height and vigour, and caused various other abnormalities. Whereas, no abnormal phenotype was observed in tobacco, cotton [79], and broccoli [80] when transformed with *Trichoderma* chitinase (*cht42*). Also, transgenic rice lines that were

developed with *cht42* gene did not show any abnormal phenotype [81].

It was reported earlier that transgenic rice over-expressing *Chi11* gene showed 15-54% reduction in sheath blight disease index [67]. Shah *et al*. [81] observed 32-62% reduction in sheath blight disease index in transgenic rice lines expressing *Trichoderma cht42* gene. Elevated chitinase activity in transgenic canola [47], strawberry [49], rice [76], tobacco [44] plants provided increased resistance to fungal diseases. However in rice and cotton the level of chitinase activity did not correlate with the level of resistance to fungal pathogens [79]. Different chitinase cDNAs of rice were isolated and were introduced into rice cultivars to develop sheath blight resistant transgenics [67], [82].

REFERENCES

[1] Mohammed S, Abd Samad A, Rahmat Z. Agrobacterium-mediated transformation of rice: constraints and possible solutions. Rice Science. 2019 May 1;26(3):133-46.

[2] Sabu S, Khanam S, Subitsha AJ. Agrobacterium-Mediated Transformation in Oryza Sativa (Rice) to Improve Crop Yield: A Review.

[3] Bennetzen JL, Ma J. The genetic colinearity of rice and other cereals on the basis of genomic sequence analysis. Current opinion in plant biology. 2003 Apr 1;6(2):128-33.

[4] Gaur, V.S., Channappa, G., Chakraborti, M., Sharma, T.R. and Mondal, T.K., 2020. 'Green revolution'dwarf gene sd1 of rice has gigantic impact.

[5] Siddiq EA, Vemireddy LR. Advances in Genetics and Breeding of Rice: An Overview. Rice Improvement. 2021:1-29.

[6] Ferrero-Serrano Á, Cantos C, Assmann SM. The role of dwarfing traits in historical and modern agriculture with a focus on rice. Cold Spring Harbor perspectives in biology. 2019 Nov 1;11(11):a034645.

[7] Jin D, Nassirou TY. Progress and perspectives of distant heterosis in Rice. Advances in plant breeding strategies: breeding, biotechnology and molecular tools. 2015:193-214.

[8] Huang X, Yang S, Gong J, Zhao Y, Feng Q, Gong H, Li W, Zhan Q, Cheng B, Xia J, Chen N. Genomic analysis of hybrid rice varieties reveals numerous superior alleles that contribute to heterosis. Nature communications. 2015 Feb 5;6(1):1-9.

[9] Sun Y, He W, Xie Y, Zhao W, Tan J, Yang X, Dang T, Dong X, Xi K, Liu Y, Hu Y. Exploiting interspecific heterosis between African rice and Asian rice. Crop Science. 2020 Sep;60(5):2343-53.

[10] Skamnioti P, Gurr SJ. Against the grain: safeguarding rice from rice blast disease. Trends in biotechnology. 2009 Mar 1;27(3):141-50.

[11] Savary, S., Willocquet, L., Pethybridge, S.J. *et al.* The global burden of pathogens and pests on major food crops. *Nat Ecol Evol* **3,** 430–439 (2019). https://doi.org/10.1038/s41559-018-0793-y

[12] Asibi AE, Chai Q, Coulter JA. Rice blast: A disease with implications for global food security. Agronomy. 2019 Aug;9(8):451.

[13] Shahriar SA, Imtiaz AA, Hossain MB, Husna A, Eaty MN. Rice blast disease. Annual Research & Review in Biology. 2020 Mar 19:50-64.

[14] Saha S, Garg R, Biswas A, Rai AB. Bacterial diseases of rice: an overview. J Pure Appl Microbiol. 2015 Mar;9(1):725-36.

[15] Jena M, Adak T, Rath PC, Gowda GB, Patil NB, Prasanthi G, Mohapatra SD. Paradigm shift of insect pests in rice ecosystem and their management strategy. ORYZA-An International Journal on Rice. 2018;55(spl):82-9.

[16] Rijal S, Devkota Y. A review on various management method of rice blast disease. Malaysian Journal of Sustainable Agriculture. 2020;4(1):14-8.

[17] Simkhada K, Thapa R. Rice Blast, A Major Threat to the Rice Production and its Various Management Techniques. Turkish Journal of Agriculture-Food Science and Technology. 2022 Mar 1;10(2):147-57.

[18] Balasubramanian V, Bell M, Sombilla M. Yield, profit, and knowledge gaps in rice farming: Causes and development of mitigation measures.

BRIDGING THE RICE YIELD GAP IN THE ASIA-PACIFIC REGION. 2000:163.

[19] Tan WZ, Zhang W, Ou ZQ, Li CW, Zhou GJ, Wang ZK, Yin LL. Analyses of the temporal development and yield losses due to sheath blight of rice (Rhizoctonia solani AG1. 1a). Agricultural Sciences in China. 2007 Sep 1;6(9):1074-81.

[20] Yellareddygari SK, Reddy MS, Kloepper JW, Lawrence KS, Fadamiro H. Rice sheath blight: a review of disease and pathogen management approaches. Journal of Plant Pathology & Microbiology. 2014 Jan 1;5(4):1.

[21] Yarasi B, Sadumpati V, Immanni CP, Vudem DR, Khareedu VR. Transgenic rice expressing Allium sativumleaf agglutinin (ASAL) exhibits high-level resistance against major sap-sucking pests. BMC Plant Biology. 2008 Dec;8(1):1-3.

[22] Jamaloddin M, Mahender A, Gokulan CG, Balachiranjeevi C, Maliha A, Patel HK, Ali J. Molecular Approaches for Disease Resistance in Rice. InRice Improvement 2021 (pp. 315-378). Springer, Cham.

[23] Mammadov J, Buyyarapu R, Guttikonda SK, Parliament K, Abdurakhmonov IY, Kumpatla SP. Wild relatives of maize, rice, cotton, and soybean: treasure troves for tolerance to biotic and abiotic stresses. Frontiers in plant science. 2018 Jun 28;9:886.

[24] Jain D, Khurana JP. Role of pathogenesis-related (PR) proteins in plant defense mechanism. InMolecular aspects of plant-pathogen interaction 2018 (pp. 265-281). Springer, Singapore.

[25] Ali S, Ganai BA, Kamili AN, Bhat AA, Mir ZA, Bhat JA, Tyagi A, Islam ST, Mushtaq M, Yadav P, Rawat S. Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. Microbiological Research. 2018 Jul 1;212:29-37.

[26] Joshi V, Joshi N, Vyas A, Jadhav SK. Pathogenesis-related proteins: role in plant defense. InBiocontrol agents and secondary metabolites 2021 Jan 1 (pp. 573-590). Woodhead Publishing.

[27] Kaur R, Kalia A, Lore JS, Sandhu JS. Antifungal effect of Trichoderma spp. β-1, 3-glucanase on Phytophthora parasitica: Hyphal morphological distortions. Journal of Phytopathology. 2020 Dec;168(11-12):700-6.

[28] Wang Y, Liu M, Wang X, Zhong L, Shi G, Xu Y, Li Y, Li R, Huang Y, Ye X, Li Z. A novel β-1, 3-glucanase Gns6 from rice possesses antifungal activity against Magnaporthe oryzae. Journal of Plant Physiology. 2021 Oct 1;265:153493.

[29] Otsuka Y, Sato K, Yano S, Kanno H, Suyotha W, Konno H, Makabe K, Taira T. GH-16 type β-1, 3-glucanase from Lysobacter sp. MK9-1 enhances antifungal activity of GH-19 type chitinase, and its glucan-binding domain binds to fungal cell-wall. Journal of Applied Glycoscience. 2022 Apr 14:jag-JAG.

[30] Patel ZM, Mahapatra R, Jampala SS. Role of fungal elicitors in plant defense mechanism. InMolecular Aspects of Plant Beneficial Microbes in Agriculture 2020 Jan 1 (pp. 143-158). Academic Press.

[31] Ramakrishna B, Sarma PV, Ankati S, Bhuvanachandra B, Podile AR. Elicitation of defense response by transglycosylated chitooligosaccharides in rice seedlings. Carbohydrate Research. 2021 Dec 1;510:108459.

[32] Gaikwad HD, Dalvi SG, Hasabnis S, Suprasanna P. Electron Beam Irradiated Chitosan elicits enhanced antioxidant properties combating resistance to Purple Blotch Disease (Alternaria porri) in Onion (Allium cepa). International journal of radiation biology. 2022 Jan 2;98(1):100-8.

[33] Vaghela B, Vashi R, Rajput K, Joshi R. Plant chitinases and their role in plant defense–a comprehensive review. Enzyme and Microbial Technology. 2022 Apr 30:110055.

[34] Saboki Ebrahim KU, Singh B. Pathogenesis related (PR) proteins in plant defense mechanism. Sci. Against Microb. Pathog. 2011;2:1043-54.

[35] El Hadrami A, Adam LR, El Hadrami I, Daayf F. Chitosan in plant protection. Marine drugs. 2010 Apr;8(4):968-87.

[36] Kumar M, Brar A, Yadav M, Chawade A, Vivekanand V, Pareek N. Chitinases—potential

candidates for enhanced plant resistance towards fungal pathogens. Agriculture. 2018 Jul;8(7):88.

[37] Kasprzewska AN. Plant chitinases-regulation and function. Cellular and Molecular Biology Letters. 2003 Jan 1;8(3):809-24.

[38] Garcia-Rubio R, de Oliveira HC, Rivera J, Trevijano-Contador N. The fungal cell wall: Candida, Cryptococcus, and Aspergillus species. Frontiers in Microbiology. 2020:2993.

[39] Datta K, Koukolikova-Nicola Z, Baisakh N, Oliva N, Datta SK. Agrobacterium-mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems. Theoretical and Applied Genetics. 2000 Apr;100(6):832-9.

[40] Nandakumar R, Babu S, Kalpana K, Raguchander T, Balasubramanian P, Samiyappan R. Agrobacterium-mediated transformation of indica rice with chitinase gene for enhanced sheath blight resistance. Biologia plantarum. 2007 Mar;51(1):142-8.

[41] Shah JM, Raghupathy V, Veluthambi K. Enhanced sheath blight resistance in transgenic rice expressing an endochitinase gene from Trichoderma virens. Biotechnology letters. 2009 Feb;31(2):239-44.

[42] Richa K, Tiwari IM, Devanna BN, Botella JR, Sharma V, Sharma TR. Novel chitinase gene LOC_Os11g47510 from indica rice Tetep provides enhanced resistance against sheath blight pathogen Rhizoctonia solani in rice. Frontiers in plant science. 2017 Apr 25;8:596.

[43] Zhang C, Huang M, Sang X, Li P, Ling Y, Zhao F, Du D, Li Y, Yang Z, He G. Association between sheath blight resistance and chitinase activity in transgenic rice plants expressing McCHIT1 from bitter melon. Transgenic Research. 2019 Aug;28(3):381-90.

[44] Saiprasad GV, Mythili JB, Anand L, Suneetha C, Rashmi HJ, Naveena C, Ganeshan G. Development of Trichoderma harzianum endochitinase gene construct conferring antifungal activity in transgenic tobacco.

[45] Akbari M, Nadaf E, Lotfi M, Tohidfar M. Transformation of Iranian melon for increasing resistance to fungal diseases. Research in Plant Sciences. 2013;1:1-3.

[46] Prasad K, Bhatnagar-Mathur P, Waliyar F, Sharma KK. Overexpression of a chitinase gene in transgenic peanut confers enhanced resistance to major soil borne and foliar fungal pathogens. Journal of Plant Biochemistry and Biotechnology. 2013 Apr;22(2):222-33.

[47] Solgi T, Moradyar M, Zamani MR, Motallebi M. Transformation of canola by chit33 gene towards improving resistance to Sclerotinia sclerotiorum. Plant Protection Science. 2015 Mar 31;51(1):6-12.

[48] Osman GH, Assem SK, Alreedy RM, El-Ghareeb DK, Basry MA, Rastogi A, Kalaji HM. Development of insect resistant maize plants expressing a chitinase gene from the cotton leaf worm, Spodoptera littoralis. Scientific reports. 2015 Dec 14;5(1):1-1.

[49] Chalavi V, Tabaeizadeh Z, Thibodeau P. Enhanced resistance to Verticillium dahliae in transgenic strawberry plants expressing a Lycopersicon chilense chitinase gene. Journal of the american society for horticultural science. 2003 Sep 1;128(5):747-53.

[50] Xu J, Zhang N, Wang K, Xian Q, Dong J, Qi X, Chen X. Chitinase Chi 2 Positively Regulates Cucumber Resistance against Fusarium oxysporum f. sp. cucumerinum. Genes. 2021 Dec 27;13(1):62.

[51] Shah MR, Mukherjee PK, Eapen S. Expression of a fungal endochitinase gene in transgenic tomato and tobacco results in enhanced tolerance to fungal pathogens. Physiology and Molecular Biology of Plants. 2010 Jan;16(1):39-51.

[52] Li W, Deng Y, Ning Y, He Z, Wang GL. Exploiting broad-spectrum disease resistance in crops: from molecular dissection to breeding. Annual review of plant biology. 2020 Apr 29;71:575-603.

[53] Geda CK, Repalli SK, Dash GK, Swain P, Rao GJ. Enhancement of Drought Tolerance in Rice through Introgression of Arabidopsis DREB1A through Transgenic Approach. J. Rice Res. 2019;7(2).

[54] Chen X, Liu P, Mei L, He X, Chen L, Liu H, Shen S, Ji Z, Zheng X, Zhang Y, Gao Z. Xa7, a new executor R gene that confers durable and broad-spectrum resistance to bacterial blight

disease in rice. Plant communications. 2021 May 10;2(3):100143.

[55] Saifi SK, Passricha N, Tuteja R, Nath M, Gill SS, Tuteja N. OsRuvBL1a DNA Helicase Boost Salinity and Drought Tolerance in Transgenic Indica Rice Raised by In-Planta Transformation (2021).

[56] Chen, W.P., Gu, X., Liang, G.H., et al. (1998). Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the bar gene as a selectable marker. Theoretical and Applied Genetics, 97, 1296-1306.

[57] Swapna, L., Khurana, R., Kumar, S.V., et al. (2011). Pollen-specific expression of Oryza sativa indica pollen allergen gene (OSIPA) promoter in rice and Arabidopsis transgenic systems. Molecular Biotechnology, 48, 49-59.

[58] Ramesh, S., Nagadhara, D., Reddy, V.D., & Rao, K.V. (2004). Production of transgenic indica rice resistant to yellow stem borer and sap-sucking insects using super-binary vectors of Agrobacterium tumefaciens. Plant Science, 166, 1077-1085.

[59] Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens* mediated transformation of indica and japonica rice varieties. Planta 199: 612–617.

[60] Japelaghi, R.H., Haddad, R., & Garoosi, G.A. (2011). Rapid and efficient isolation of high quality nucleic acids from plant tissues rich in polyphenols and polysaccharides. Molecular Biotechnology, 49, 129-137.

[61] Russell DW, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 2001.

[62] Bolar, J.P., Norelli, J.L., Wong, K.W., et al. (2000). Expression of endochitinase from Trichoderma harzianum in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology, 90, 72-77.

[63] Ling, K.C., & Ou, F.H. (1969). Standardization of international race numbers of P. oryzae. Phytopathology, 59, 339-342.

[64] Dong OX, Ronald PC. Genetic engineering for disease resistance in plants: recent progress and future perspectives. Plant physiology. 2019 May;180(1):26-38.

[65] Conrath U. Systemic acquired resistance. Plant signaling & behavior. 2006 Jul 1;1(4):179-84.

[66] Kamthan A, Chaudhuri A, Kamthan M, Datta A. Genetically modified (GM) crops: milestones and new advances in crop improvement. Theoretical and Applied Genetics. 2016 Sep;129(9):1639-55.

[67] Sridevi, G., Parameswari, C., Rajamuni, P., & Veluthambi, K. (2006). Identification of hemizygous and homozygous transgenic rice plants in T_1 generation by DNA blot analysis. *Plant Biotechnology*, 23, 531-534.

[68] Koul B. Genetically Modified (GM) Crops Harbouring Bacillus thuringiensis (BT) Gene (S) to Combat Biotic Stress Caused by Insect Pests. InPhyto-Microbiome in Stress Regulation 2020 (pp. 21-61). Springer, Singapore.

[69] Saida L, Shravya M, Reddy VP. Standardization of efficient regeneration protocol and agrobacterium mediated genetic transformation in RPBio-226 variety of rice (Oryza sativa). Crop Research (0970-4884). 2022 Jan 1;57.

[70] Chern M, Canlas PE, Fitzgerald HA, Ronald PC. Rice NRR, a negative regulator of disease resistance, interacts with Arabidopsis NPR1 and rice NH1. The Plant Journal. 2005 Sep;43(5):623-35.

[71] Campbell, M.A., Fitzgerald, H.A., & Ronald, P.C. (2002). Engineering pathogen resistance in crop plants. Transgenic Research, 11, 599-613.

[72] Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J. Genetically engineered resistance to Fusarium head blight in wheat by expression of Arabidopsis NPR1. Molecular Plant-Microbe Interactions. 2006 Feb;19(2):123-9.

[73] Fan W, Dong X. In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid–mediated gene activation in Arabidopsis. The Plant Cell. 2002 Jun;14(6):1377-89.

[74] Kim YW, Youn JH, Roh J, Kim JM, Kim SK, Kim TW. Brassinosteroids enhance salicylic acid-mediated immune responses by inhibiting BIN2 phosphorylation of clade I TGA

transcription factors in Arabidopsis. Molecular Plant. 2022 May 6.

[75] Leadbeater A, Staub T. 13 Exploitation of Induced Resistance: A Commercial Perspective. Induced resistance for plant defense: a sustainable approach to crop protection. 2014 Oct 20:300.

[76] Li P, Pei Y, Sang X, Ling Y, Yang Z, He G. Transgenic indica rice expressing a bitter melon (Momordica charantia) class I chitinase gene (McCHIT1) confers enhanced resistance to Magnaporthe grisea and Rhizoctonia solani. European journal of plant pathology. 2009 Dec;125(4):533-43.

[77] Zhang C, Huang M, Sang X, Li P, Ling Y, Zhao F, Du D, Li Y, Yang Z, He G. Association between sheath blight resistance and chitinase activity in transgenic rice plants expressing McCHIT1 from bitter melon. Transgenic Research. 2019 Aug;28(3):381-90.

[78] Bolar JP, Norelli JL, Wong KW, Hayes CK, Harman GE, Aldwinckle HS. Expression of endochitinase from Trichoderma harzianum in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology. 2000 Jan;90(1):72-7.

[79] Emani C, Garcia JM, Lopata-Finch E, Pozo MJ, Uribe P, Kim DJ, Sunilkumar G, Cook DR, Kenerley CM, Rathore KS. Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from Trichoderma virens. Plant Biotechnology Journal. 2003 Sep;1(5):321-36.

[80] Mora AA, Earle ED. Resistance to Alternaria brassicicola in transgenic broccoli expressing a Trichoderma harzianum endochitinase gene. Molecular breeding. 2001 Aug;8(1):1-9.

[81] Shah JM, Raghupathy V, Veluthambi K. Enhanced sheath blight resistance in transgenic rice expressing an endochitinase gene from Trichoderma virens. Biotechnology letters. 2009 Feb;31(2):239-44.

[82] Zhang C, Huang M, Sang X, Li P, Ling Y, Zhao F, Du D, Li Y, Yang Z, He G. Association between sheath blight resistance and chitinase activity in transgenic rice plants expressing McCHIT1 from bitter melon. Transgenic Research. 2019 Aug;28(3):381-90.