Journal of Biotechnology 166 (2013) 114–121

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Transgenic indica rice lines, expressing *Brassica juncea* Nonexpressor of pathogenesis-related genes 1 (*BjNPR1*), exhibit enhanced resistance to major pathogens

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ARTICLE INFO

Article history:
Received 28 January 2013
Received in revised form 3 April 2013
Accepted 21 April 2013
Available online 9 May 2013

Keywords:
Agronomic traits
Brassica juncea
Herbicide tolerance
Nonexpressor of pathogenesis-related gene
Pathogen resistance
Transgenic rice

ABSTRACT

Brassica juncea Nonexpressor of pathogenesis-related genes 1 (*BjNPR1*) has been introduced into commercial indica rice varieties by *Agrobacterium*-mediated genetic transformation. Transgenic rice plants were regenerated from the phosphinothricin-resistant calli obtained after co-cultivation with *Agrobacterium* strain LBA4404 harbouring Ti plasmid pSB111-*bar*-*BjNPR1*. Molecular analyses confirmed the stable integration and expression of *BjNPR1* in various transgenic rice lines. Transgenes *NPR1* and *bar* were stably inherited and disclosed co-segregation in subsequent generations in a Mendelian fashion. Homozygous transgenic rice lines expressing *BjNPR1* protein displayed enhanced resistance to rice blast, sheath blight and bacterial leaf blight diseases. Rice transformants with higher levels of *NPR1* revealed notable increases in plant height, panicle length, flag-leaf length, number of seeds/panicle and seed yield/plant as compared to the untransformed plants. The overall results amply demonstrate the profound impact of *BjNPR1* in imparting resistance against major pathogens of rice. The multipotent *BjNPR1*, as such, seems promising as a prime candidate gene to fortify crop plants with durable resistance against various pathogens.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important staple food crops of the world, and serves as the major source of nutrition for more than half of the global population. However, a number of challenges have to be overcome to attain the goal of increased rice production in a sustainable manner to meet the demand of ever growing population. In rice, more than 70 diseases caused by fungi, bacteria and viruses have been identified. Three major diseases, viz. rice blast caused by *Magnaporthe grisea*, sheath blight (SB) caused by *Rhizoctonia solani* and bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *Oryzae* have been considered as the most devastating diseases (Ou, 1985). These diseases are responsible for causing annual yield losses of upto 50% of rice productivity (Datta et al., 2002). Development of resistant cultivars and application of pesticides have been used extensively for controlling these diseases. However, in case of SB the desired level of genetic variability is lacking in the cultivated rice and its wild relatives (Bonman et al., 1992). As such, it is imperative to develop alternative disease control strategies for providing durable and broad-based resistance. One among such strategies is the induced resistance which has emerged as a potential supplement to the conventional crop protection measures (Taheri and Tarighi, 2010). In plants, systemic acquired resistance (SAR) is the most extensively studied type of induced resistance.

Nonexpressor of pathogenesis-related genes 1 (*NPR1*) – also known as the non-inducible immunity 1(*NIM*1)/Salicylic acid insensitive (*SAI*) – has been found to act as the master regulator of salicylic acid-mediated SAR signaling. The *NPR1* protein contains an ankyrin repeat and a BTB/POZ domain, and functions downstream to the SA (Cao et al., 1997). In the cytoplasm, *NPR1* remains as an oligomer in an 'inactive' state. Owing to biphasic changes in the redox potential in the cytoplasm caused by SA treatment or pathogen challenge, the *NPR1* oligomers get monomerized. The active monomers translocate to the nucleus because of the presence of bipartite nuclear localization signal (Cao et al., 1998), and interact with TGA type b-ZIP transcription activators leading to their increased DNA-binding activity to cognate *cis*-elements in the promoters of defense-related genes (Despres et al., 2000). These transcription factors bind to the *as*-1 elements (TGACG) in the

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http://dx.doi.org/10.1016/j.jbiotec.2013.04.016

regulatory sequences and enhance the transcription of genes controlled by these promoters (Niggeweg et al., 2000). Fu et al. (2012) reported that *NPR1* paralogues NPR3 and NPR4 act as SA receptors which bind SA with different affinities, and function as adaptors of the Cullin 3 ubiquitin E3 ligase to mediate *NPR1* degradation in an SA-regulated manner. Furthermore, *NPR1* suppresses hypersensitive response (HR) due to $CUL3^{NPR3}$-mediated degradation of *NPR1*, and enables *NPR1* to accumulate in the margin of the HR to restrict the spread of programmed cell death and establish SAR.

Validation for the key role played by *NPR1* in various defense networks has been obtained by a number of over-expression studies. Constitutive expression of *Arabidopsis thaliana NPR1* (*AtNPR1*) in different crops caused increased resistance to both fungal and bacterial diseases (Cao et al., 1998; Friedrich et al., 2001; Quilis et al., 2008; Lin et al., 2004; Makandar et al., 2006; Wally et al., 2009). Meur et al. (2006) reported that the *BjNPR1* protein was found to cause redox-regulation in mustard akin to that of *AtNPR1* in *Arabidopsis*. In the present investigation, *BjNPR1* has been introduced into commercial rice varieties and transgenic plants were evaluated against major fungal and bacterial diseases. Transgenic rice lines expressing *BjNPR1* exhibited higher levels of resistance against blast, SB and BLB diseases.

2. Materials and methods

2.1. Construction of Ti-super binary vector containing BjNPR1 and bar expression cassettes

An 1.74 kb cDNA coding region of *BjNPR1* (AY667498) was cloned between CaMV35S promoter and polyA terminator at *Bam*HI site of pRT100 vector (Topfer et al., 1987). Later, the expression unit of *BjNPR1* was excised and cloned into *Hind*III site of the intermediate vector pSB11 *bar* (Yarasi et al., 2008). The resultant recombinant vector, pSB11*bar*-CaMV35S-*BjNPR1*-polyA, was mobilized into *A. tumefaciens* strain LBA4404 by triparental mating as described (Ramesh et al., 2004), and the co-integrate super-binary vector was designated as pSB111-*bar*-*BjNPR1*.

2.2. Agrobacterium-mediated transformation and regeneration of transgenic plants

Two popular indica rice cultivars, Chaitanya and Samba Mahsuri (SM) obtained from the Directorate of Rice Research (DRR), Hyderabad, were employed for genetic transformation. *Agrobacterium*-mediated genetic transformation experiments were carried out using LBA4404 strain harbouring pSB111-*bar*-*BjNPR1* super-binary vector according to Yarasi et al. (2008). Transgenic plants (30–40 day old) along with untransformed controls were tested for their tolerance to the herbicide 0.25% Basta (Ramesh et al., 2004).

2.3. PCR, Southern and northern blot analysis

Genomic DNA was isolated from the Basta tolerant and untransformed control plants using the method of McCouch et al. (1988). PCR analysis was carried out using the primers; 5′-TTG GTC GTA CTC AAG CTC-3′ and 5′-GTT GTC TGG TTG CTT GAG-3′ for *BjNPR1*, and 5′-CTA CCA TGA GCC CAG AAC G-3′ and 5′-GTT TGC GCG CTA TAT TTT GTT-3′ for *bar*-*nos*. DNA isolated from the untransformed control plants was used as the negative control and intermediate vector was used as the positive control. For Southern blot analysis, 10–12 μg of genomic DNA was digested with *Bam*HI, electrophoresed on a 0.8% agarose gel and subsequently transferred to an N^+ Nylon membrane (Amersham Biosciences) and fixed by exposing to UV (1200 μJ for 60 s) in an UV cross linker (Sambrook and Russell, 2001). The blot was probed with α-^{32}P dCTP employing ready to go random primer DNA labelling kit (Amersham Biosciences) and an 1.7 kb *BjNPR1* and 560 bp *bar* coding regions were used as probes. Similarly, northern blot analysis was carried out using total RNA isolated from the untransformed control as well as transformants. About 10 μg of RNA was separated on 1.4% denaturing agarose gel and an 1.7 kb *BjNPR1* coding region was used as probe.

2.4. Western blot analysis of BjNPR1 lines

Samples of *BjNPR1* rice transformants and untransformed control leaf tissue were homogenized in 50 mM Tris–HCl buffer (pH 9.0). The extract was centrifuged at 5000 × *g* for 20 min at 4 °C and the supernatant was collected. The protein concentration of the supernatant was estimated using Bradford reagent as per the manufacturer's instructions. To reduce disulfide bonds, 50 mM Dithiothreitol (DTT) was added to the protein extracts together with 2× sample buffer (125 mM Tris–HCl, pH 6.8; 5% SDS; 25% glycerol and 0.4% bromophenol blue). The protein samples (20 μg) were heated at 60 °C for 10 min and were subjected to 10% SDS–PAGE. Following electrophoresis, the separated proteins were transferred onto nitrocellulose N^- membrane by electroblotting, and western blotting was done as described by Bharathi et al. (2011) using polyclonal anti-*BjNPR1* antibodies supplied by M/s Varada Biotech, Bhubaneshwar, India.

2.5. Enzyme Linked Immunosorbent Assay (ELISA) analysis

Wells of the microtitre plate were coated with 1 μg of crude protein extract of untransformed control as well as transgenic plants and kept for overnight at 37 °C. ELISA was performed using anti-*BjNPR1* primary antibody (1:1000 dilutions) and secondary antibody (1:10,000 dilutions) according to Bharathi et al. (2011).

2.6. Fungal and bacterial bioassays

Bioassays were carried out under standard controlled conditions in the glass house at the Directorate of Rice Research (DRR), Rajendranagar, Hyderabad. T_1, T_2, T_3 and T_4 generations of *BjNPR1* transformants along with untransformed control plants and susceptible rice varieties were used in fungal and bacterial infections. Resistance exhibited by the plants was scored based on a scale of 0–9 as per the Standard Evaluation System (SES) of the International Rice Research Institute (1996).

2.6.1. Evaluation of transgenics for leaf blast resistance

Selfed seeds of *BjNPR1* transformants along with the respective untransformed controls, HR12 (susceptible) and IR64 (resistant) were germinated in seedling beds. Fifteen-day old seedlings were subjected to *M. grisea* strain IC9 (International Race C, Group 9, origin DRR, Hyderabad) infection using fully infected HR12 rice leaves. Disease index was recorded after 15 days of infection as described by Ling and Ou (1969) using the SES scale (1996). Further, the infection was continued for a period of 30 days under similar conditions and photographs were taken.

2.6.2. Evaluation of transgenics for sheath blight resistance

Transgenic plants, untransformed control plants along with the susceptible variety (IR 50) were inoculated with *R. solani* colonized typha pieces at the maximum tillering stage (90–100 old plants). Typha pieces (8–10 pieces/plant) with the mycelium of the *R. solani* isolate DRR were placed between the tillers, 5 cm above the water level, ensuring that the tillers were tightly secured, and allowed them to grow to maturity. Disease symptoms were recorded after 20 days of infection. Disease index was calculated by counting both