

Research Application Summary

**Molecular analysis of over-expression of NPR1 gene in putative transgenics of
Brassica juncea plants**

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Abstract

Brassica accounts for nearly 27% of vegetable oil requirements of India. Its production however is constrained by Alternaria blight (*Alternaria brassicae*). The absence of resistance genes within crossable germplasm of *Brassica juncea* necessitates the use of genetic engineering strategies to develop genetic resistance against this pathogen. Potential of regulatory genes for developing fungi- and bacteria-resistant transgenic plants has been demonstrated in *Arabidopsis* and other plants, one such regulatory gene being *NPR1*. *Brassica juncea* *NPR1* (*BjNPR1*) gene was over-expressed in *Brassica juncea*. Transformation of hypocotyls of *B. juncea* var. Varuna with the *BjNPR1* gene under the control of constitutive *CaMV35S* promoter using *Agrobacterium tumefaciens* strain EHA105 resulted in about 1.4% of transformation efficiency. The integration of *BjNPR1* into the plant genome was confirmed by PCR using *NPTII* primers, in which 90% of the plants analyzed were found to be PCR positive. Moreover, over-expression of the *BjNPR1* transcript was shown in 60% of the total plants analyzed by RT-PCR using *NPR1* primers.

Key words: *Brassica juncea*, NPR1, over-expression, PCR

Résumé

Brassica survient à près de 27% des besoins en huile végétale de l'Inde. Toutefois sa production est limitée par la brûlure Alternaria (*Alternaria brassicae*). L'absence de gènes de résistance au sein du germoplasme de *Brassica juncea* nécessite l'utilisation du génie génétique pour développer une résistance génétique contre ce pathogène. Le potentiel de gènes régulateurs pour le développement de plantes transgéniques résistantes aux champignons et bactéries a été démontré chez *Arabidopsis* et d'autres plantes, l'un de ces gènes régulateurs étant le *NPR1*. Le gène *NPR1* (*BjNPR1*) de *Brassica juncea* a été surexprimé chez *Brassica juncea*. La transformation des hypocotyles de *B. juncea* var. Varuna avec le gène *BjNPR1* sous contrôle de *CaMV35S* en utilisant la souche EHA105 d'*Agrobacterium tumefaciens* a donné environ 1,4% d'efficacité de transformation. L'incorporation de *BjNPR1* dans le génome de la plante a été confirmée par PCR en utilisant des amorces *NPTII*, dans lesquelles 90% des plantes analysées se sont révélées positives à

la PCR. Par ailleurs, la surexpression du gène BjNPR1 a été montrée dans 60% des plantes analysées par RT-PCR en utilisant des amorces NPR1.

Mots clés: *Brassica juncea*, NPR1, sur-expression, PCR

Background

Oil seeds constitute the second largest agricultural commodity in India after cereals accounting for nearly 5% of gross national product (GDP) and 10% of the value of all agricultural products. India is the third largest edible oil economy in the world, next to U.S. and China and occupies a distinct position in terms of both area and diversity of cultivated oilseed crops, which contributes to about 13% of the world's edible oil supply and 25.5% of the oil production of the country.

Among the oilseeds, *Brassica juncea* (L.), commonly known as Indian mustard or rapeseed-mustard, occupies a prominent position in the country contributing nearly 21.6% and 23.1% to the total oilseed cropped area and production, respectively. The crop accounts for nearly one-third of the oil produced in India, making it the country's second most important edible oil next to groundnut, and is considered as a 'cash crop' (Kumar *et al.*, 2008). Its oil content typically varies between 36% and 42%, of which, average oil recovery is approximately 34% to 35% (Srinivasan, 2005). Once the oil is extracted, the remaining part of the seed is used to produce rapeseed/mustard meal, an important source of cattle and poultry feed which represents a significant source of oil meal in the country, supplying on average about 3 to 3.2 million tones of meal annually (Pahariya and Mukherjee, 2007).

India is however, not able to meet the edible oil requirement for its vast population (Kumar *et al.*, 2008). The average yield per hectare of the crop is very low (958 kg/ha) when compared to the world average (1300 kg/ha) due to mostly the effect of insect pests and diseases. The most important disease of the crop is Alternaria blight (*Alternaria brassicae*), which accounts for about 57% of yield loss. *Alternaria brassicae* produces lesions surrounded by chlorotic areas on the leaves and stems causing reduction in photosynthetic areas, defoliation and early induction of senescence (Tewari, 1991). Plant breeders have been trying albeit without success so far to develop disease resistant lines for many years through conventional plant breeding methods. Unfortunately, there is no source of resistance against this pathogen among the sexually compatible relatives of *Brassica juncea*. Thus the use of genetic engineering strategies is essential to develop genetic resistant against this pathogen.

Potential of regulatory genes for developing fungi- and bacteria-resistant transgenic plants have been demonstrated in *Arabidopsis*, one such regulatory gene being *NPR1*. The *NPR1* gene of *Arabidopsis* activates the transcriptional factors of PR proteins and other defense genes and confers broad spectrum resistance to bacterium *Pseudomonas syringae* and fungus *Peronospora parasitica* when constitutively expressed in *Arabidopsis* (Chern *et al.*, 2001). Resistance in such transgenic plants is not associated with any adverse plant phenotypes, thus making *NPR1* an extremely interesting candidate for agriculture application.

The objective of this study was to evaluate over-expression of NPR1 gene in putative transgenics of *Brassica juncea* plants.

Study description

The study was conducted at Indian Agricultural Research Institute (IARI), Pusa campus, New-Delhi. The *NPR1* cDNA clone of *Brassica juncea*, with GenBank No. of DQ359129 and total cDNA length of 2137 bp with coding length of 1782 bp starting at 136 bp and ending at 1917 bp; and cloned in pTriplex plasmid (Grover *et al.*, 2006) was available in the lab. The binary vector pBI121 harboring the reporter GUS driven by the CaMV 35S promoter was used for cloning *NPR1* cDNA under the control of 35S promoter. *Agrobacterium tumefaciens* (strain EHA105) was used for plant transformation.

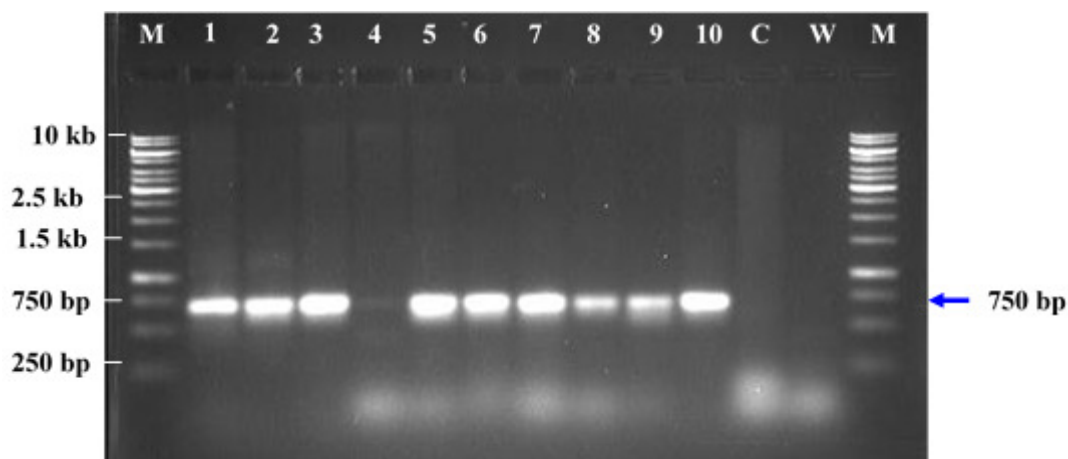
For molecular analysis, total genomic DNA and RNA was extracted from young leaf tissues of putative *Brassica juncea* transgenic plantlets and untransformed wild type *Brassica juncea* plants using CTAB method (Murray and Thompson, 1980) and Trizol method (Sigma-Aldrich, USA), respectively. The presence of the transgene in the putative transgenic plantlets was finally analyzed by PCR and RT-PCR for its integration and expression, respectively. Total genomic DNA was extracted using CTAB method from the leaf tissues of the plantlets and was checked on 0.8% agarose gel. Subsequently, PCR with gene-specific *nptII* (Forward: 5'-AGGCGATAGAAGGCGATGCGC-3' and Reverse: 5'-CAATCGGCTGCTCTGATGCCG-3') primers obtained from Bangalore Genei, employing an annealing temperature of 63°C, was carried out to confirm the integration of the insert into the plant genome. Genomic DNA extracted from untransformed *Brassica juncea* was also included as a negative control in the polymerase chain reaction with *nptII* primers. The amplification was done by setting the thermo cycler machine at 94°C for 5 min (initial denaturation) followed by 30 cycles of each 94°C for 30 sec (denaturation), 63°C for 30 sec (annealing) and 72°C for 1 min (extension) with one cycle of final extension at 72°C for 7 min and 4°C for infinity to preserve the amplified products. The PCR products were then visualized under UV light after electrophoresis in 1% ethidium stained agarose gel along with 1 kb DNA ladder (MBI Fermentas, USA) as a marker and picture was taken in the gel document.

For the analysis of the expression of the transgene in the putative *Brassica juncea* transgenic plantlets, total RNA was extracted from young leaf tissues of the plantlets using the Trizol method. Reverse transcriptase PCR (RT-PCR), SuperScript™ III (One-Step RT-PCR System with Platinum® *Taq* DNA polymerase), provided by InvitroGen, was carried out using gene-specific *NPR1* primers (Forward: 5'-TACTGACCTCCTGAAACGTGAG-3' and Reverse: 5'-TGTCCCCGGTAACTCTGTAACAC-3'). RNA isolated from untransformed *Brassica juncea* and RT-PCR reaction mixture without any RNA (with water) were included as positive and negative controls, respectively. The thermal cycler was programmed at 55°C for 30 min (cDNA synthesis) and 94°C for 2 min (Inactivation of reverse transcriptase) immediately followed by 40 cycles of each 94°C for 15 sec (denaturation), 60°C for 30 sec (annealing) and 68°C for 1 min/kb (extension) with one cycle of final extension at 68°C for 5 min.

Result and discussion

The PCR products from the samples of putative *Brassica juncea* transgenic plantlets, electrophoresed on 1% agarose gel, showed a single sharp band of 750 bp; while in the untransformed plant DNA, the band was missing (Fig. 1). Out of 10 plants analyzed, 9 plants were found to be PCR positive. The amplified *NPR1* cDNA clone from the genomic DNA of kanamycin resistant shoots using the *nptII* gene-specific primers clearly confirmed the integration of the transgene into the genome of the putative plantlets, but not from the genomic DNA of the untransformed plant. PCR with gene-specific *NPR1* primers was not done to differentiate the transformants from the untransformed plantlets of *B. juncea* as the gene is naturally present in the genome of the plant.

In order to confirm the expression of the gene in the transgenics, RT-PCR was done by using equal amounts of RNA (1 µg) from the putative transgenics using *NPR1* primers. RNA isolated from non-transformed plants was also included in the RT-PCR with equal amount (1 µg) as a positive control in order to compare the amplification products between the transgenics and the control. The RT-PCR products were then checked on 1% agarose gel and were observed under UV conditions. Ten selected putative transgenics were analyzed, all of which showed the expected 1 kb amplified fragment including from the positive control, demonstrating transcriptionally active *NPR1* gene in the plant genome (Fig. 2). However, the amplification of the RT-PCR products from all of the 10 analyzed putative transgenics was not similar. The RT-PCR analysis showed that *NPR1* is over-expressed two-three fold in six of the transgenics (sample 1, 3, 5, 6, 7 & 8) as compared to the non-transgenic plant



M: 1 kb DNA ladder (Marker); Lanes 1-10: PCR products of 10 DNA samples from putative transgenic explants of **B. juncea** showing 750 bp amplification fragment of DNA; C: PCR amplification of DNA from untransformed **B. juncea** used as a control; W: PCR amplification without DNA (that is with water only) used as a control

Figure 1. PCR analysis of genomic DNA (from putative *Brassica juncea* plants) with *nptII* primers observed on 1% agarose gel

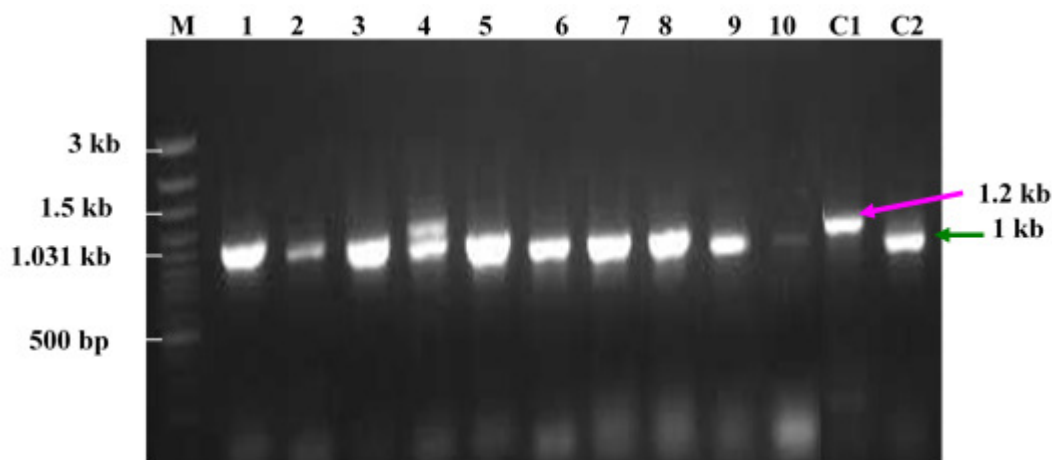


Figure 2. RT-PCR analysis of 10 putative transgenic *B. juncea* plants with NPR1 primer checked on 1% agarose gel. M: Marker (Gene Ruler 100bp Ladder Plus); Lanes 1-10: RT-PCR products of amplified cDNA showing a fragment length of 1 kb; C1: Genomic DNA used as control showing amplified fragment of 1.2 kb; C2: Control with RNA from untransformed *B. juncea*

(Fig. 2). Primers of *NPR1* were chosen in such a way so as to span a 200 bp intron in the DNA. So, when DNA was used as a template for RT-PCR, the product was 1.2 kb as compared to RNA amplified 1 kb product (Fig. 2, C₁). Therefore, the two bands observed in one of the transgeics (Fig. 2, Lane 4) indicate that there is DNA contamination in the RNA sample.

The results indicate that *NPR1* gene can be over-expressed in *B. juncea* so as to develop resistance against multiple pathogens. Over-expression of the gene has no detrimental effect on the plant phenotypes as it was demonstrated in *Arabidopsis thaliana* (Chern *et al.*, 2001), because the gene gets activated only when there is pathogen infection. Therefore, manipulation of *NPR1* gene, which regulates and activates several downstream pathogenesis-related and other defense genes, is crucial for the development of fungal and bacterial resistant transgenic plants.

From these results, it can be concluded that the gene is over-expressed in the putative transgenic plantlets although the level of expression varied among different transgenics. The presence of 1 kb band from the positive control, on the other hand, indicates that the gene is naturally expressed in the wild plants of *B. juncea*.

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