

# Combined expression of a barley class II chitinase and type I ribosome inactivating protein in transgenic *Brassica juncea* provides protection against *Alternaria brassicae*

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**Abstract** *Alternaria* leaf spot caused by *Alternaria brassicae*, or *A. brassicicola*, is one of the major fungal diseases of *Brassica juncea* (Indian mustard). To develop resistance against this fungal disease, the barley antifungal genes class II chitinase (AAA56786) and type I ribosome inactivating protein (RIP; AAA32951) were coexpressed in Indian mustard via *Agrobacterium*-mediated transformation. The stable integration and expression of transgenes in T<sub>0</sub> plants were confirmed by Southern blot and Western analysis. The transgenic lines showing inheritance in Mendelian fashion (3:1) were further evaluated by in vitro studies and under greenhouse conditions for resistance to the *A. brassicae* fungal pathogen. The transgenic plants showed up to 44% reduction in *A. brassicae* hyphal growth in in vitro antifungal assays. In green house screening, the transgenic plants sprayed with *A. brassicae* spores showed resistance through delayed onset of the disease and restricted number, size, and expansion of lesions as compared to wild type plants. These results indicate that the expression of chitinase and RIP from a heterologous source in *B. juncea* provide subsequent protection against *Alternaria* leaf spot disease and can be helpful in increasing the production of Indian mustard.

**Keywords** *Brassica juncea* · *Alternaria* leaf spot · *Alternaria brassicae* · Fungal resistance · Transgenic plants · Chitinase and ribosome inactivating proteins

## Abbreviations

BA 6-Benzylaminopurine  
NAA  $\alpha$ -Naphthaleneacetic acid  
MS Murashige and Skoog

## Introduction

*Brassica juncea* (Indian mustard) is a major oilseed crop of the Brassicaceae family. It is cultivated in over 6 million hectares of land on the Indian subcontinent as well as in many other countries around the world. Mustard alone fulfils nearly 27.1% of vegetable oil requirements of India and is estimated to meet 41% of the demand by 2020 (Yadav and Singh 1999). Due to increased demand for edible oil and biofuels, the cultivation of oilseeds crops has gained significant importance (Kumar 1999). Unfortunately, the productivity of this crop has been hampered by several biotic and abiotic factors (Grover and Pental 2003). One of the major constraints affecting the productivity of mustard is the *Alternaria* leaf spot disease caused by *Alternaria brassicae* and *A. brassicicola* (Singh 1990; Meena et al. 2010). The estimated yield loss has been reported up to be as high as 46% (Kolte et al. 1987). This fungus produces symptoms throughout all parts of the plant, i.e. on cotyledons, leaves, leaf petiole, stem, inflorescence, siliquae and seeds. The disease causes the formation of dark colored necrotic lesions with concentric rings on leaves and pods. In severe cases, the vitality of the crop is badly affected and the siliquae fails to set healthy seeds. Resistance to *Alternaria* blight is not available in the cultivated *Brassica* species (Sharma et al. 2002). Wild crucifers such as *Sinapis alba* (Kolte et al. 1991), *Camelina sativa*, *Capsella bursa-pastoris* (Conn et al. 1988), and *Neslia paniculata* (Tewari and Conn 1993) have shown a

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high degree of resistance to *A. brassicae*, however transfer of disease resistance from wild sources through conventional breeding is very difficult due to sexual incompatibility with cultigens and limited gene exchange in hybrids involving resistant wild species. Consequently, conventional breeding programs have not been successful in terms of conveying fungal resistance (Yadav and Singh 1999). In light of these difficulties, a genetic engineering approach was taken to transfer antifungal proteins from different plant species to *B. juncea*.

Pathogenesis-related (PR) proteins are well known to provide resistance to fungal infection in various cases. Hydrolytic enzymes, such as chitinases and  $\beta$ -1,3-glucanases have been shown to inhibit fungal growth by degrading structural polysaccharides in fungal cell walls (Broglie et al. 1991; Leah et al. 1991; Zhu et al. 1994; Jach et al. 1995). Ribosome inactivating proteins (RIP) are a group of plant proteins that possess 28S rRNA *N*-glycosidase activity, which leads to the inactivation of foreign ribosomes in distantly related species and in other eukaryotes, including fungi (Stirpe et al. 1992). The constitutive expression of a single antifungal gene has been shown to enhance resistance against fungal pathogens in several crops such as tobacco, wheat, maize, rice, and *B. juncea* (Logemann et al. 1992; Jach et al. 1995; Bliffeld et al. 1999; Datta et al. 2001; Bieri et al. 2003; Mondal et al. 2003; Shin et al. 2008; Girhepuje and Shinde 2011). However, if enzymes from two gene families are combined, they can act synergistically with improved effect on fungus infection. When a class II chitinase and a type 1 RIP from barley were coexpressed in tobacco, increased protection against *Rhizoctonia solani* was demonstrated in vivo (Jach et al. 1995). The combined gene expression also significantly enhanced protection against *A. alternata* and *Botrytis cineria*. Furthermore, the combined expression of modified maize RIP and rice basic chitinase in rice significantly enhanced protection against the sheath blight pathogen *R. solani* (Kim et al. 2003).

In this study, to check the synergistic effect of antifungal genes, barley class II chitinase (AAA56786) and RIP (AAA32951) were used in combination to engineer

*B. juncea* var. Pusa Jaikisan for enhanced protection against the fungus *A. brassicae*.

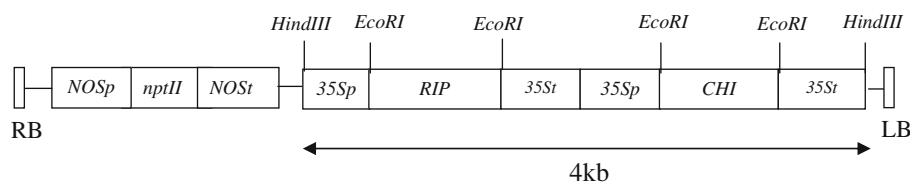
## Materials and methods

### Plant material, bacterial strains and vectors

Seeds of a commercially grown cultivar of *B. juncea*, Pusa Jaikisan, were obtained from the Department of Plant Breeding, CCS Haryana Agricultural University, Hisar, India. The disarmed *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pGJ42 (Fig. 1) was used for transformation studies. The plasmid GJ42 contains two antifungal genes, barley chitinase (AAA56786) and RIP (AAA32951) under the control of CaMV35S promoter and a selectable marker gene, neomycin phosphotransferase (*nptII*) driven by *nos* promoter. The *Agrobacterium* culture was grown overnight in liquid YEM (Yeast extract, 1.0 g l<sup>-1</sup>; mannitol, 10 g l<sup>-1</sup>; NaCl, 0.1 g l<sup>-1</sup>; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g l<sup>-1</sup>; K<sub>2</sub>HPO<sub>4</sub>, 0.5 g l<sup>-1</sup>; pH 7.2) containing 50 mg l<sup>-1</sup> kanamycin and 20 mg l<sup>-1</sup> rifampicin at 28°C on a rotatory shaker at 200 rpm.

### *Agrobacterium*-mediated transformation of *B. juncea*

The Brassica transformation was done as described by Prasad et al. (2000). Hypocotyls of *B. juncea* cv. Pusa Jaikisan were used as explants. The explants were pre-cultured on filter paper moistened with liquid MS basal medium (Murashige and Skoog 1962) containing 0.5  $\mu$ M BA for 36 h. The pre-cultured explants were infected and co-cultivated with *A. tumefaciens* on MS medium with 2 mg l<sup>-1</sup> BA and 2 mg l<sup>-1</sup> NAA for 1 day. The transformed explants were selected on medium with 250 mg l<sup>-1</sup> cefotaxime and 30 mg l<sup>-1</sup> kanamycin (this concentration was chosen based on kanamycin toxicity tests from our preliminary experiments). The shoots were regenerated in two 2 weeks cycles. In the first cycle, the regeneration medium contained 250 mg l<sup>-1</sup> cefotaxime and 30 mg l<sup>-1</sup> kanamycin was used. In the second cycle, the kanamycin concentration was reduced to 20 mg l<sup>-1</sup> for efficient shoot regeneration. The



**Fig. 1** T-DNA region of binary vector pGJ42 used for *Agrobacterium*-mediated transformation. Abbreviations: RB right border, LB left border, 35S 35Sp promoter and 35St terminator, NOS nopaline

synthase promoter or terminator, *nptII* neomycin phosphotransferase, *RIP* ribosome inactivating protein, *CHI* chitinase

rooted, transformed shoots were established in pots in a greenhouse at 25–28°C with 16 h light and 65% humidity and grown to maturity to collect T<sub>1</sub> seeds.

#### Molecular analysis of transformants

##### *PCR and Southern hybridization*

Total genomic DNA was extracted from fresh leaves of putative transformants (T<sub>0</sub>) and wild type plants using the CTAB (Cetyl trimethyl ammonium bromide) method (Rogers and Bendich 1988). Putative transformants were analyzed by PCR for the presence of the *nptII* gene. The 0.54 kb coding region of *nptII* was amplified using 20 bp oligonucleotide primers (Forward 5'-CCACCATGATA TTCGGCAAC-3' and Reverse 5'-GTGGAGGCTATTTCGG CTA-3'). To ensure that reagents were not contaminated, DNA from wild type plant was included in the experiments. The amplified products were separated by electrophoresis on 1% (w/v) agarose gel and visualized by staining with ethidium bromide (Sambrook et al. 1989). Five to ten micrograms of DNA isolated from transformed and wild type plants were digested with *HindIII*, separated on 0.9% (w/v) agarose gel, and blotted on positively charged nylon membrane (Roche Molecular Biochemical). It was then hybridized with dCT-[P<sup>32</sup>]-labeled probe (PCR amplified *nptII* gene fragment of 0.75 kb) and checked with autoradiography. The probe was made using random labeling method and Southern hybridization was performed according to Sambrook et al. (1989).

##### *Western analysis*

Total protein was extracted from fresh leaves of transformed and wild type plants in a phosphate buffer (pH 7.0) as described by Jach et al. (1995) and estimated using the Bradford method (Bradford 1976). A 20 µg of protein was resolved on 12% SDS-PAGE and transferred to Hybond PVDF membrane (Biometra) using an electro-transfer system (Bangalore Genei, India). The membrane was blocked in Phosphate buffer saline (PBS) with 5% non-fat dried milk (Amresco) at 37°C for 2 h. After blocking, the membrane was incubated with primary antibodies (barley chitinase and RIP, 1:500 dilutions) for 2 h at room temperature followed by three washes with PBST (PBS with 0.01% (v/v) Tween 20) for 10 min each. Next, alkaline phosphatase conjugated secondary antibodies (Goat anti-rabbit IgG, 1:10,000 dilution, Bangalore Genei) were added for 1 h. The membrane was again washed with PBST four times for 10 min each at room temperature. The proteins were detected after adding NBT-BCIP (Nitroblue tetrazolium-Bromochloroindolylphosphate) substrate and developing in the dark.

##### *Segregation analysis*

To determine the pattern of inheritance of chitinase and RIP genes, T<sub>1</sub> seeds from each self-pollinated transformed line were germinated on MS medium containing 30 mg l<sup>-1</sup> kanamycin for 2 weeks. The green seedlings were considered as resistant whereas non-germinated/brown seedlings as sensitive. The kanamycin resistant plants of each transgenic line were transferred to soil and further analyzed by semi-quantitative RT-PCR (Qiagen one step RT-PCR kit) in T<sub>2</sub> plants.

#### **Evaluation of transgenic plants for resistance to *A. brassicae***

##### *In vitro* antifungal assay

The *in vitro* inhibitory effect of soluble protein extracted from leaves of kanamycin resistant *B. juncea* transgenic plants on *A. brassicae* hyphal growth was assayed as described by Mondal et al. (2003). Wells made on antibiotic assay agar plates (Himedia) were first filled with 40 µl of spore suspension (10<sup>4</sup> spores ml<sup>-1</sup>) and incubated overnight at 30°C. After incubation, each well was differently treated with 40 µl each of transgenic leaf extract (100 µg protein/40 µl), autoclaved transgenic leaf extract, protein extraction buffer, and commercial chitinase (1.5 µg/40 µl; Sigma) solution. The plates were incubated at 25°C. The protein from wild type plants (100 µg total protein) and protein extraction buffer (Phosphate buffer, pH-7.0) served as controls. The radial growth of *A. brassicae* was recorded every 24 h for 5 days. The percent inhibition of hyphal growth was calculated as the ratio of the diameter of the fungal colony in treatment to the diameter of the fungal colony in wild type plants, multiplied by 100.

##### Greenhouse screening

*A. brassicae* spore culture (Accession no-ITCC 5095) was obtained from Indian Type culture collection (ITCC), Division of Plant Pathology, IARI, New Delhi. This culture was used as a test organism. The fungal culture was maintained on potato dextrose agar (PDA) medium at 22 ± 1°C. The spore suspension was prepared as described by Kanrar et al. (2002). Four plants (T<sub>1</sub>) from each of four transgenic lines and wild type were assayed for fungus infection. Two to three month old plants were inoculated with *A. brassicae* spore suspension adjusted to 10<sup>4</sup> spores ml<sup>-1</sup> using a haemocytometer and 200 ml of spore suspension was sprayed on each plant using an atomizer. The plants were kept in the greenhouse at 25°C with 75–85% relative humidity. The data on lesions per leaf (average of four

leaves per plant) and individual lesion diameter (in mm) were recorded 10 days after inoculation. Percent disease protection was calculated as the number of lesions per leaf in control plants minus the number of lesions per leaf in transgenic plants divided by the number of lesions per leaf in control plants, multiplied by 100.

The different disease parameters such as incubation period, latent period, necrotic lesions, lesions size and lesions per leaf were taken to compare the disease reaction of control and transgenic plants. The incubation period was considered to be from the inoculation time to the appearance of pin head/small lesions, whereas the latent period was considered to be up to the appearance of concentric rings in the lesion. Observations were recorded daily for 15 days after inoculation.

### Statistical analysis

The experiments were repeated twice. Mean  $\pm$  SE of diameter of lesions and percentage disease protection were calculated using Microsoft Excel software version 2003. The disease protection in in vitro inhibition and greenhouse assays were analyzed by student *T*-test. The segregation analysis in the progeny plants was validated by  $\chi^2$  test.

## Results and discussion

### Generation of transgenic plants

About 36% pre-cultured explants showed regeneration on selection medium with kanamycin. A total of 15 putative transgenic plants were transferred to soil in the greenhouse, out of which ten survived and grew to maturity as independent lines.

### Molecular analysis of transformants

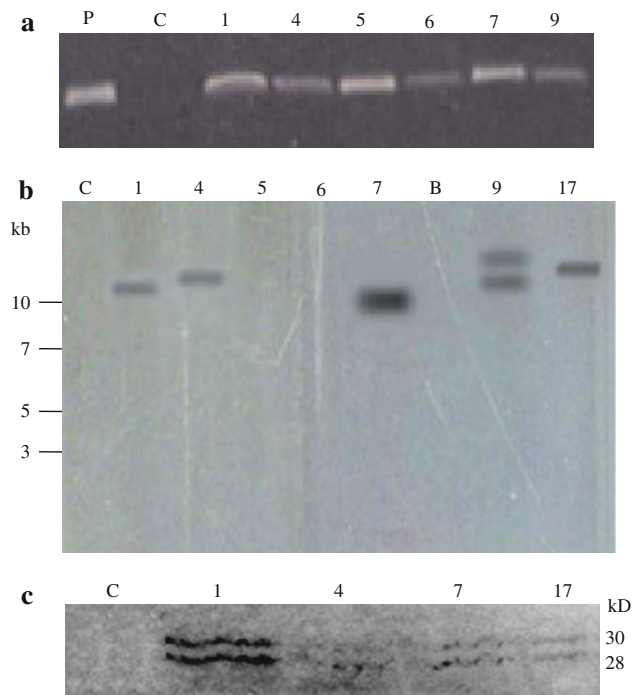
#### PCR and Southern blot analysis

The putative transgenic plants were initially screened by PCR for the presence *nptII* gene. 70% of putative transformants of *B. juncea* showed amplification of the 0.54 kb *nptII* gene. However, no amplification was detected in wild type plants (Fig. 2a). In order to check the integration and copy number of transgenes in the Brassica genome, seven PCR positive plants were analyzed by Southern blotting. The genomic DNA was digested with *HindIII* and hybridized with a 0.75 kb *nptII* probe. Because *HindIII* cuts the entire 35S-RIP/35S-CHI region of pGJ42 cassette as a 4.0 kb internal fragment, the integration will be confirmed only if the detected band sizes are higher than 4.0 kb. Out of seven plants, five putative transgenic plants

showed the presence of one to two bands of size more than 4.0 kb, suggesting the integration of *nptII* gene at one to two independent positions in the plant genome (Fig. 2b). This showed that these transgenic plants were derived from independent transformation events. The hybridization pattern showed that four plants had a single copy and one had two copies of the T-DNA in their genome. DNA isolated from wild type plants did not hybridize with the *nptII* probe.

### Western blotting

The expression of chitinase and RIP proteins in transgenic plants was confirmed by Western blotting (Fig. 2c). The total soluble proteins from four Southern positive transgenic lines showing a single copy T-DNA insertion and wild type plants were immunoblotted and detected using antibodies specific for barley chitinase and RIP. All four transgenic lines showed the expression of both chitinase (28 kD) and RIP (30 kD) proteins. Transgenic line 1

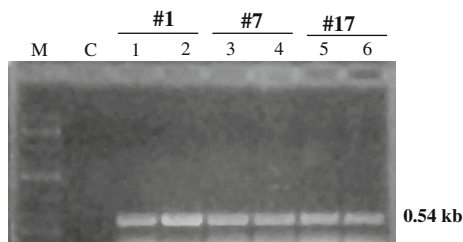


**Fig. 2** Analysis of transgenic plants by PCR, and Southern and Western blotting. **a** PCR analysis of control and transgenic plants with *nptII* marker gene: *P* plasmid DNA; *C* DNA from wild type plant; lane 3–8 DNA from transgenic lines. **b** Southern blot analysis of *HindIII* digested genomic DNA from independent transgenic lines and probed with the PCR amplified *nptII* fragment (0.75 kb): *C* wild type plant DNA digested with *HindIII*; lane 2–9 DNA from transgenic lines digested with *HindIII*; *B* blank lane. **c** Western analysis of independent transgenic lines with chitinase and RIP specific antibodies: *C* protein from untransformed plant; lanes 1, 4, 7, 17 transgenic lines protein

**Table 1** Segregation pattern of neomycin phosphotransferase (*nptII*) gene in T<sub>1</sub> progenies on kanamycin

Transgenic line	No. of seeds on kanamycin	Resistant seedlings	Sensitive seedlings	Observed ratio	$\chi^2$ value*
1	65	50	15	3.3:1	0.126
4	60	44	16	2.8:1	0.088
7	60	45	15	3.0:1	0.000
9	44	28	16	1.8:1	3.029
17	64	49	15	3.2:1	0.0825

\* The difference from expected 3:1 segregation is not significant at  $P < 0.05$  except transgenic line 9



**Fig. 3** Semi-quantitative RT-PCR of T<sub>2</sub> plants with *nptII* gene. Lane 1 DNA ladder (10 kb); C control plant cDNA; lane 1–2 (line 1); lane 3–4 (line 7); lane 5–6 (line 17)

showed the highest expression, whereas, lines 4, 7, and 17 had lower level of chitinase and RIP expression. No expression of the barley chitinase and RIP was detected in the wild type plants.

#### Segregation analysis

The inheritance of transgenes to the next generation is shown in Table 1. Out of five transgenic lines, four segregated in a 3:1 ratio on kanamycin, while line 9 showed a 2:1 segregation pattern, which may be due to abnormal insertion of transgenes in the plant genome. RT-PCR analysis showed mRNA expression of the *nptII* gene in two randomly selected kanamycin resistant plants from each of three transgenic lines 1, 7, and 17 (Fig. 3).

#### Evaluation of transgenic plants for resistance to *A. brassicae*

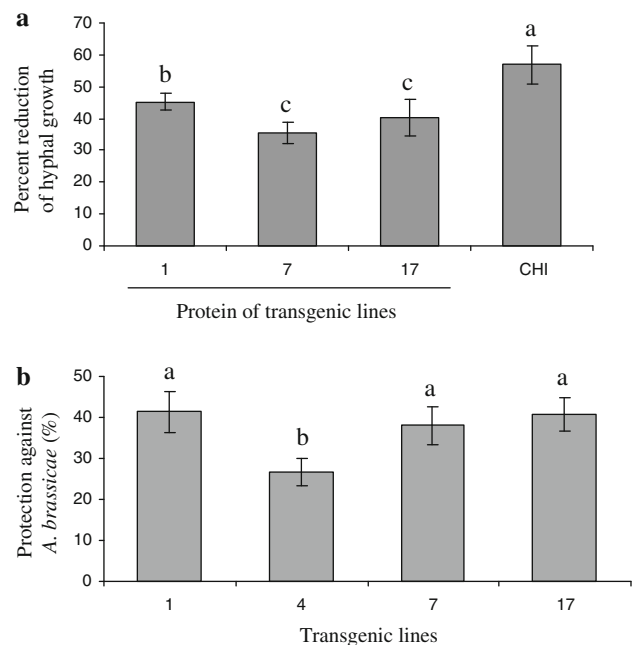
##### In vitro antifungal assay

The crude protein extracted from three independent transgenic mustard lines (1, 7, and 17) were evaluated for their effect on the fungal hyphal growth in vitro. The total water-soluble proteins (100  $\mu\text{g}/40 \mu\text{l}$ ) from three independent transgenic plants restricted the fungal hyphal growth ranging from 36 to 44% over the wild type plants (Fig. 4a). Among the three independent lines tested, line 1 showed the maximum suppression of hyphal growth. The inhibition

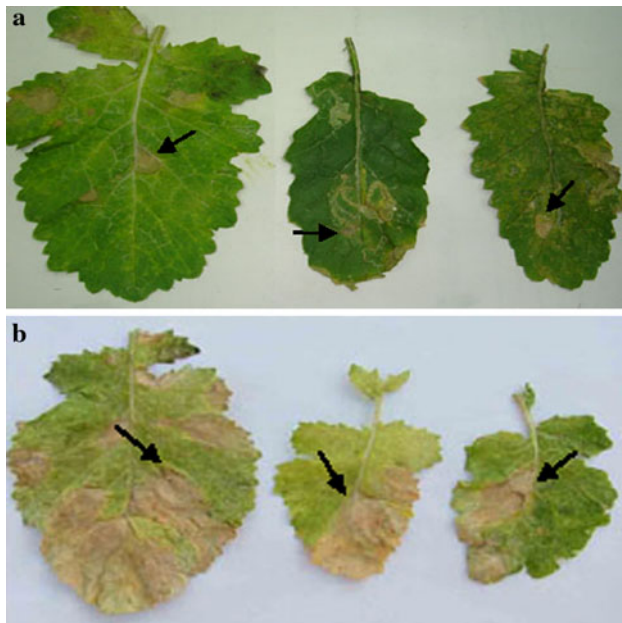
of fungal growth by different transgenic plants was in correlation to the levels of chitinase and RIP expression as revealed by western analysis. There was no such inhibition of fungal growth in the presence of either boiled extract or only extraction buffer. The observed inhibition of hyphal growth of *A. brassicae* may be due to degradation of chitin, a major structural polysaccharide of growing hyphae, by chitinase and inactivation of fungal ribosomes by RIP (Logemann et al. 1992; Leah et al. 1991).

##### Greenhouse screening of transgenics

In greenhouse screening, the characteristic lesions formed after spraying *A. brassicae* spores ( $10^4$  spores/ml of H<sub>2</sub>O)



**Fig. 4** Analysis of inhibition in hyphal growth of *A. brassicae* in transgenic lines. **a** Percent inhibition in hyphal growth of *A. brassicae* by protein extracts of *B. juncea* transgenic lines overexpressing barley chitinase and RIP proteins and commercial chitinase at 1.5  $\mu\text{g}/40 \mu\text{l}$  (CHI). **b** The percent protection from *A. brassicae* by kanamycin resistant T<sub>1</sub> plants of transgenic *B. juncea* under greenhouse bioassay. Data is mean  $\pm$  SE. Columns with different letters are significantly different at  $P < 0.05$



**Fig. 5** Infection of *A. brassicae* on transgenic and non-transformed (control) plants under green house conditions. **a** Minute lesions on leaves from GJ42 transformed plant, **b** Concentric rings on leaves of non-transformed plants and also leaf tip drying and yellowing of leaves

were less in number on leaves of transgenic plants (<10 lesions per leaf) compared to wild type plants (>15 lesions per leaf). Individually, transgenic plants showed variability in disease protection (Fig. 4b). The lesions produced by *A. brassicae* in transgenic plants were restricted to 2.5–4.0 mm in diameter without formation of concentric rings, whereas in non-transgenic plants the average lesion size varied between 10 and 16 mm in diameter with concentric rings (Fig. 5). In transgenic plants, combined antifungal activities of chitinase and RIP proteins prevented the formation of this kind of concentric ring pattern by dissolution of growing hyphae and inhibition of the spread of fungus across the tissue layers. Different host-pathogen systems have shown resistance in the form of reduced lesion size due to restricted growth of fungal pathogens (Bliffeld et al. 1999; Broglie et al. 1991; Jach et al. 1995).

Other parameters such as incubation period (from time of inoculation to appearance of pinhead/small size lesions), latent period (from time of inoculation to appearance of concentric rings in the lesions), and symptoms such as leaf tip drying and necrotic lesions are shown in Table 2 to demonstrate the effect of *A. brassicae* on the whole plant. The incubation period of *A. brassicae* was delayed by 3 days in transgenic plants compared to wild type plants. The maximum number of small size lesions reached the concentric ring stage 10 days after inoculation in transgenic plants as compared to wild type plants where it took 4–5 days to reach the concentric ring stage. The latent

**Table 2** Different parameters of disease resistance in transformed and non-transformed plants in response to *A. brassicae* infection

Parameters	Non-transformed plants	Transformed plants
Incubation period (days)	3	6
Latent period (days)	4	8
Symptoms		
Leaf tip drying	7–8 day after inoculation	11–12 days after inoculation
Necrotic lesions	Characteristic yellow ring	Smaller in size

period was also longer (8 days) in transgenic plants than wild type plants (4 days). It has been shown under field conditions in conventional breeding programs that susceptible rapeseed mustard variety ‘Parkash’ has shorter incubation and latent periods than resistant ‘Towar’ cultivar (Saharan and Kadian 1983). In the present study, transgenic plants showed a significant delay in disease development due to longer incubation and latent periods. Similarly, Kanrar et al. (2002) reported that transgenic *B. juncea* plants expressing the rubber tree laccin, have exhibited resistance against *A. brassicae*. A longer incubation period of 120 h in transgenic plants rather than 60 h in control plants was the major factor for delay in disease progression in transgenic plants. The latent period was also longer in transgenic plants as compared to wild type plants under greenhouse conditions. The delay in onset of disease is attributed to the antifungal activity in the transgenic plants. Previously, the constitutive expression of chitinase in *B. juncea* has shown resistance to *Alternaria* blight by delaying the onset of disease and reducing the number of lesions (Mondal et al. 2003, 2007). However, the amount of transgenic leaf protein used in in vitro fungal growth inhibition was eight fold higher (400 µg/20 µl) (Mondal et al. 2007) than those used in the present study (100 µg/40 µl). Similarly, Broglie et al. (1991) showed a delay in infection of *R. solani* in chitinase expressing transgenic tobacco. Since the hydrolytic chitinase enzyme will degrade the fungal cell wall and RIP’s 28S rRNA *N*-glycosidase activity leads to the inactivation of foreign ribosomes of distantly related species and of the other eukaryotes including fungi, combined expression of chitinase and RIP is expected to even further increase resistance. Jach et al. (1995) reported that combined expression of barley chitinase and RIP in tobacco significantly enhanced protection against the fungal pathogen *R. solani* compared to the protection level obtained by the expression of only a single barley transgene. In rice, the coexpression of modified RIP and rice basic chitinase provided tolerance

to *R. solani* but failed to provide tolerance to *Bipolaris oryzae* and *Magnaporthe grisea* (Kim et al. 2003). Our results are consistent with those reported in previous studies. However, in the present study, without a direct comparison of plants with single transgenes, it is difficult to ascertain whether the combined effect of chitinase and RIP genes is better than either gene alone.

In conclusion, the constitutive co-expression of chitinase and RIP in *B. juncea* confers protection against *Alternaria* leaf spot by arresting the growth of the pathogen *A. brassicae*, and thereby decreases disease severity in the transgenic plants compared to control plants. However, further experiments are needed to evaluate fungal resistance of transgenic lines under field conditions and to test for resistance to other pathogens.

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