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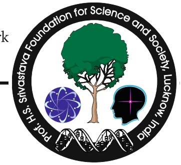

RESEARCH ARTICLE

Expression of an endochitinase gene from *Trichoderma virens* confers enhanced tolerance to *Alternaria* blight in transgenic *Brassica juncea* (L.) czern and coss lines

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Abstract An endochitinase gene '*ech42*' from the biocontrol fungus '*Trichoderma virens*' was introduced to *Brassica juncea* (L). Czern and Coss via *Agrobaterium tumefaciens* mediated genetic transformation method. Integration and expression of the '*ech42*' gene in transgenic lines were confirmed by PCR, RT-PCR and Southern hybridization. Transgenic lines (T_1) showed expected 3:1 Mendelian segregation ratio when segregation analysis for inheritance of transgene '*hpt*' was carried out. Fluorimetric analysis of transgenic lines (T_0 and T_1) showed 7 fold higher endochitinase activity than the non-transformed plant. Fluorimetric zymogram showed presence of endochitinase (42 kDa) in crude protein extract of transgenic lines. In detached leaf bioassay with fungi *Alternaria brassicae* and *Alternaria brassicicola*, transgenic lines (T_0 and T_1) showed delayed onset of lesions as well as 30–73 % reduction in infected leaf area compared to non-transformed plant.

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Keywords *Trichoderma virens* · '*ech42*' gene · *Brassica juncea* · *Agrobacterium* -mediated transformation · Endochitinase activity · Fungal resistance

Introduction

Brassica juncea L. Czern and Coss (Indian mustard) is one of the major oil seed crops cultivated in many countries, including India, where mustard is cultivated in around 6 million hectares of land (Yadava and Singh 1999). This crop is highly susceptible to various fungal pathogens and insects, whereas bacterial and viral diseases have little effect on its yield (Abdel-Farida et al. 2009). *Alternaria* blight caused by *A. brassicae* (Berk.) Sacc. (Black leaf spot disease) and *A. brassicicola* (Grey leaf spot disease) is one of the major diseases of this crop causing up to 47 % yield losses (Singh et al. 1999; Meena et al. 2002). With the increasing demand for brassica oil especially in developing countries, it is necessary to minimize the yield losses caused by biotic stresses. Genetic engineering is an alternative for developing disease resistant plants where the resistance source is not available or there are sexual compatibilities between the source and the cultivar. (Grover and Gowthaman 2003; Chhikara et al. 2012).

Genes from the mycoparasitic *Trichoderma* spp. have been used to impart tolerance to biotic and abiotic stresses in many crops (Nicolas et al. 2014). Among the *Trichoderma* genes transferred to plants, an endochitinase of 42 kDa has been very popular due to its broad spectrum of activity against many fungal pathogens. Endochitinases are known to degrade fungal chitin by hydrolyzing the glycosidic bond between chitin monomers. In addition, the endochitinases also release chitin oligomers which act as elicitor molecules triggering broad spectrum plant defense (Emani et al. 2003).

There are very few reports on development of transgenic *Brassica* species for resistance to fungal pathogens. For example, *B. napus* transformed with two genes *sporamin* and *chitinase PjChi-1* (derived from sweet potato and *Paecliomyces javanicus*, respectively) showed enhanced resistance against both *Plutella xylostella* and *Sclerotinia sclerotiorum* (Liu et al. 2011). Wu et al. (2009) introduced a plant defensin gene *Ovd*, cloned from *Orychophragmus violaceus* (L.) into *B. napus*, and the transgenic plants showed resistance to *S. sclerotiorum*. Transgenic *B. napus* expressing *B. napus* mitogen-activated protein kinase (*BnMPK4*) showed resistance to *S. sclerotiorum* (Wang et al. 2009). Chitinase and glucanase gene from tomato have been introduced into *B. juncea* and the transgenic plants showed resistance to *A. brassicae* (Mondal et al. 2003, 2007). We have earlier cloned and transferred an endochitinase gene from *T. virens* to tobacco and tomato and the transgenic lines exhibited enhanced tolerance to some fungal pathogens (Shah et al. 2010).

In the present study, our objectives were to express an endochitinase '*ech42*' gene from *Trichoderma virens* in transgenic *B. juncea* lines and to assess tolerance of transgenic lines against two fungal pathogens, *A. brassicicola* and *A. brassicae*, causing *Alternaria* blight.

Materials and methods

Plant material

Seeds of Indian mustard (*Brassica juncea* L. Czern and Coss) cv. Pusa Jaikisan from Indian Agriculture Research Institute (IARI), New Delhi were used for the transformation experiments.

Bacterial strain and plasmid

Agrobacterium tumefaciens strain LBA4404 harboring binary vector pCAMBIA1301:*ech42* containing the chitinase coding region (GenBank Acc.No. EU035808) (Shah et al. 2010) was used for transformation of *B. juncea*.

Transformation of *B. juncea*

Seeds of *B. juncea* were germinated aseptically in vitro on MS (Murashige and Skoog 1962) medium. The cotyledonary petioles from 5 day old seedlings were used as explants and pre-cultured for 2 day on SRM i.e. shoot regeneration medium [MS medium supplemented with 1 mg l^{-1} BA (6 -Benzyl amino purine) and 1 mg l^{-1} Kn (Kinetin)]. Transformation of *B. juncea* with *Agrobacterium tumefaciens* strain LBA4404 harboring binary vector pCAMBIA1301:*ech42* was carried out as described earlier by Kamble et al. (2013). Putative transformed plants (T_0) were self-pollinated and their seeds were germinated on MS medium containing 2.5 mg l^{-1} hygromycin. The surviving seedlings (T_1) resistant to hygromycin was counted and data analyzed using the Chi-square test to determine the number of functional '*hpt*' gene loci in the *B. juncea* genome.

Molecular analysis of putative transgenic plants

Polymerase chain reaction (PCR)

Genomic DNA was isolated from 25 plants each from 25 randomly selected T_0 lines obtained from independent transformation events and one non-transformed plant (control) according to Dellaporta et al. (1983). PCR was carried out according to Shah et al. (2010). PCR analysis was also carried out with 5 T_1 plants each from 5 different T_1 lines to confirm the stable integration of transgene.

Reverse transcription PCR (RT-PCR)

Transcription of '*ech42*' gene in PCR positive T_0 lines was confirmed by RT- PCR. Total RNA from 10 transformed lines as well as one non-transformed (control) plant was isolated using Tri-reagent. One µg of DNase-treated and purified RNA was taken for cDNA synthesis using affinity script multi temperature cDNA synthesis kit (Stratagene, USA). Two µl of this reaction mix was used for PCR amplification of '*ech42*'and a housekeeping gene 'actin' (Shah et al. 2010).

Southern blot hybridization

Southern blot hybridization was carried out to confirm the integration and copy number of '*ech42*' gene in transgenic *B. juncea* lines. Genomic DNA (50 µg) from 5 randomly selected PCR positive T_0 lines were digested with H*ind*III (NEB) and size separated on 1 % agarose gel at 25 V for 16 h. PCR amplified product of '*ech42*' gene (1.2 kb) from plasmid pCAMBIA1302:*ech42* was used for probe preparation using DIG-DNA labeling kit (Roche Biochemicals, Germany). Pre-hybridization, hybridization, washing and detection were carried out using chemilluminescent detection system (Roche Biochemicals, Germany).

Fig. 1 Development of transgenic *B. juncea* with '*ech42*' gene (**a**) Cotyledon with petiole cultured on shoot regeneration medium (**b**) Explants showing regeneration on selection medium after infection with *Agrobacteria*. (**c**) Multiple shoots growing on selection medium (**d**) Elongated shoot transferred to rooting medium (**e**) Shoot with well developed roots (**f**) Transgenic plants transferred to soil for hardening (**g**) Hardened plants in pots showing flowering and pod setting

Endochitinase activity and fluorimetric zymogram

Endochitinase activity in transgenic plants (each from different T_0 and T_1 lines) was measured by fluorimetric assay and fluorimetric zymogram was performed for visualization of chitinase activity as described earlier by Shah et al. (2010). All the reagents used were from Sigma and assay was repeated at least twice with three replicates.

Detached leaf bioassay

Cultures of *Alternaria brassicae* (ITCC No. 5097) and *A. brassicicola* (ITCC No. 1707) were obtained from the Indian Type Culture Collection, Division of Plant Pathology, IARI, New Delhi. Bioassay for sensitivity of transgenic lines against the fungal pathogens were done as described earlier (Shah et al. 2010). Three independent experiments with three replicates were carried out and the data was analyzed using Analysis of Variance (ANOVA) and Pearson's correlation using Microcal™ Origin pro 6.1.

Results

Transformation of *B. juncea* with '*ech42*' gene

Prior to infection with *Agrobacterium*, cotyledonary petioles were pre-cultured on SRM for 2 days (Fig. 1a).

Table 1 Transformation efficiency (%) of *B. juncea* cv. Pusa jaikisan following co-cultivation with *A. tumefaciens* LBA4404 with '*ech42*' gene

Batch No.	No. of explants co-cultivated	No. of PCR positive plants	Transformation (%)
1	96	2	2.1
2	96	4	4.2
3	96	4	4.2
4	100	3	3.0
5	86	2	2.3
6	96	3	3.1
7	96	2	2.1
8	150	3	3.0

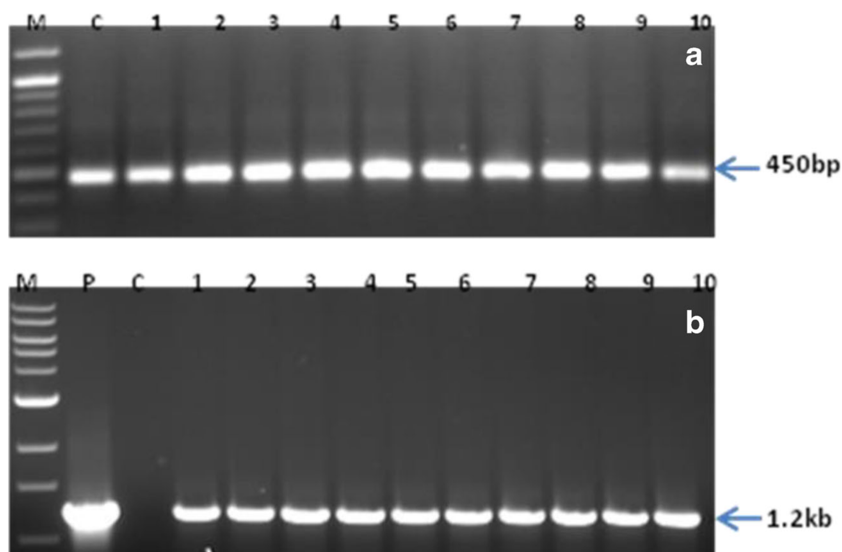

Fig. 2 RT-PCR analysis of transgenic *B. juncea* lines (T_0) (**a**) actin as 'house- keeping' gene (**b**) an '*ech42*' gene. Lane M- 100 bp ladder (**a**), 1 kb ladder (**b**), C-control (non-transgenic) plant, P- positive control- pCAMBIA1301-*ech42* plasmid, Lane 1–10 -Transgenic lines

Followed by infection with *Agrobacterium*, the explants were transferred to selection medium. Cotyledonary petioles showed initiation of shoot at the proximal end after 15–20 days of infection (Fig. 1b). The regenerated shoots were subcultured 3-4 times on selection medium i.e. SRM supplemented with 2.5 mg l^{-1} hygromycin (Fig. 1c). Several shoots were bleached at this stage. Hygromycin resistant shoots were transferred to rooting medium (Fig. 1d, e) and well rooted plants were hardened in paper cups (Fig. 1f). Total 8 independent transformation events were carried-out and 25 hygromycin resistant lines were obtained. Some plants showed reduction in leaf size and stunted growth compared to other transgenic plants, even though they produced seeds. Five plants, each from 5 different T_0 lines showing high endochitinase activity were allowed to grow in growth chamber, selfed and seeds collected for raising T_1 generation (Fig. 1g). The average transformation efficiency obtained ranged from 2.1–4.2 % (Table 1).

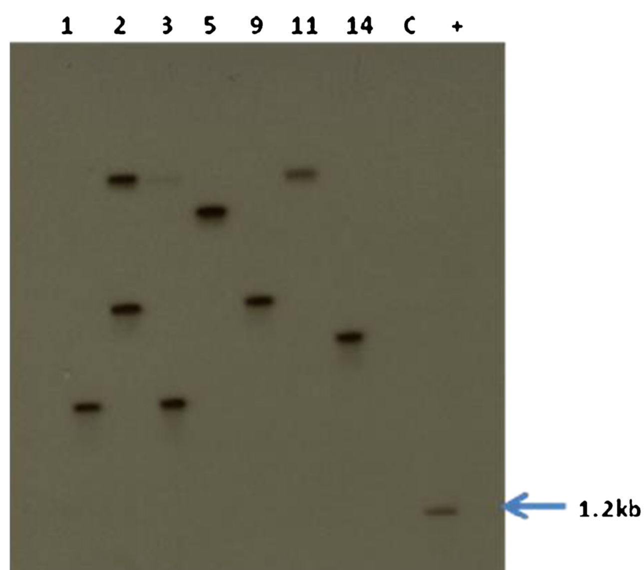

Fig. 3 Southern blot hybridization of transgenic *B. juncea* lines (T_0) - 1, 2, 3, 5, 9, 11 and 1, C- control (non-transformed) plant, (+) - positive control - *Hind*III digested PCR product of '*ech42* gene

Molecular analysis of transgenic plants

PCR and RT-PCR

Out of 25 putative transgenic T_0 lines, 20 lines showed presence of expected size PCR products confirming integrations of '*ech42*' (1.2 kb) (Fig. S1). These lines were further tested for endochitinase activity. PCR analysis of five T_1 lines also showed presence of '*ech42*'-gene (Fig. S2). RT-PCR results confirmed expression of

Table 2 Inheritance of hygromycin resistance (*hpt*) in T_1 generation of transgenic *B. juncea* lines

Line	Hyg^R seedlings	Hyg^S seedlings	χ^2 test (3:1)
Non-transformed	0	20	-
1	15	6	0.142
3	17	5	0.06
5	14	6	0.26
11	32	13	0.363
14	37	13	0.027

At $p \le 0.05$ and $n = 1$, χ^2 value is 3.84, hence χ^2 value from all lines were found to be not significant ($Hyg^{R:}$ Hygromycin resistant; $Hyg^{S:}$ Hygromycin sensitive)

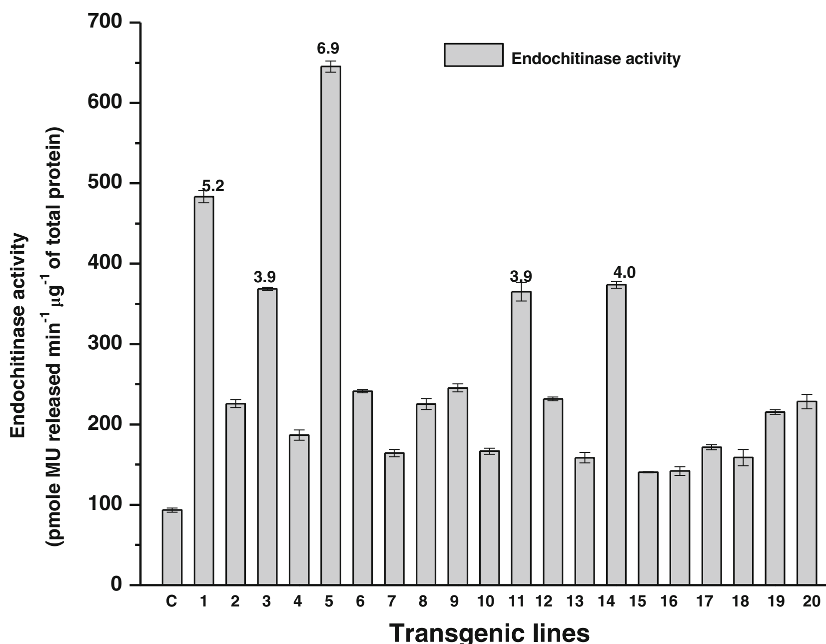

Fig. 4 Fluorimetric assay for transgenic *B. juncea lines* (T_0). Error bars indicates ± standard error of means. The number above the error bar represents fold increase in the endochitinase activity over the control plant. The values were statistically significant at $P \leq 0.05$ over the control (non-transgenic) plant

'actin' as well as '*ech42*' gene in 10 randomly selected PCR positive T_0 lines (Fig. 2a, b).

Southern blot hybridization

Seven randomly selected T_0 lines of *B. juncea* were subjected to Southern hybridization. Each transgenic line had a single band except line 2, where two copies of the gene were seen. Non-transformed (control) plants did not show any hybridization signal to the probe. All the transgenic lines showed a band of more than 5.3 kb as expected (Fig. 3).

Segregation analysis of T_0 progeny

The healthy seedlings (T_1) surviving on selection medium containing 2.5 mg l^{-1} of hygromycin obtained by germinating seeds of T_0 lines were classified as hygromycin resistant and pale seedlings were classified as hygromycin sensitive. Segregation ratio of 3:1 in T_1 generation was seen at $P \leq 0.05$, $n = 1$ (Table 2).

Table 3 Fluorimetric assay for transgenic *B. juncea* T_1 lines for measuring the level of endochitinase activity

lines	Endochitinase activity (pmol MU released min^{-1} µg^{-1} of total protein ± SE)	Fold increase over non-transformed T_1 plants
Control	85.5 ± 1.2	1
1	470.3 ± 1.4	5.5
3	369.3 ± 1.6	4.3
5	573.3 ± 2.6	6.7
11	374.0 ± 2.5	4.3
14	363.3 ± 3.9	4.2

Endochitinase activity in T_0 and T_1 plants and fluorimetric zymogram

Transgenic lines (T_0 and T_1) showed enhanced endochitinase level compared to non-transformed *B. juncea* plants. T_0 lines showed 1.5–6.9 fold higher endochitinase activity (Fig. 4) while T_1 lines showed 4.2–6.7 fold higher endochitinase activity compared with non-transformed plant (Table 3). Total protein of 10 T_0 and 4 T_1 *B. juncea* lines were separated on SDS-PAGE gel. After renaturation, the enzyme was probed with the fluorogenic substrate, [4MU-β-(glcNAc)$_3$]. Fluorescence at position of 42 kDa was detected under UV transilluminator (Fig. 5 a, b).

Detached leaf bioassay

Five T_0 and T_1 transgenic lines (line 1, 3, 5, 11 and 14) showing high endochitinase activity were challenged with two fungi, *A. brassicola* and *A. brassicae*. The transgenic lines showed delay in lesion formation compared to control plant (Fig. 6). In control plants, lesion was formed within 2 days after incubation with the fungus while transgenic plants showed lesion formation after 4–5 days. When challenged with *A. brassicicola*, T_0 lines showed 34–73.7 % reduction in lesion area and there was a strong negative correlation between endochitinase activity and % lesion area in T_0 lines (R = − 0.90) (Fig. 7a). T_1 lines also showed 31–65 %

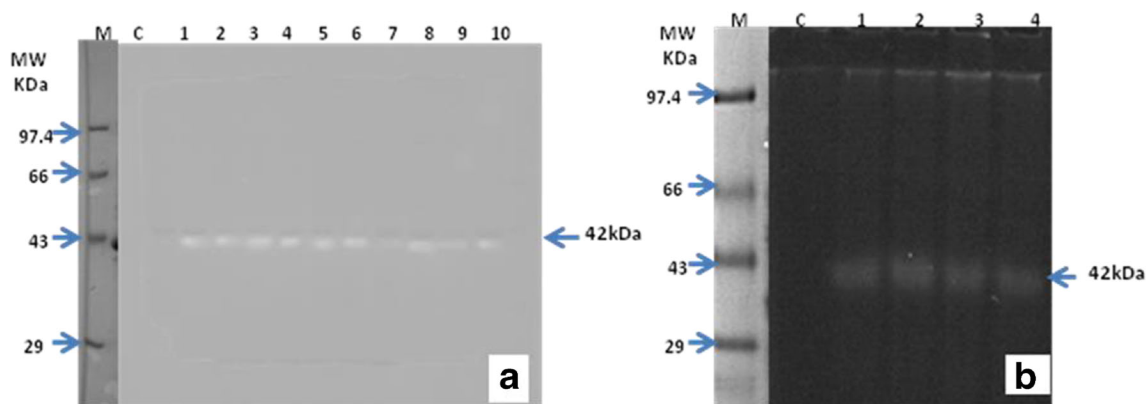

Fig. 5 Fluorimetric zymogram for detection of 42 kDa endochitinase in transgenic *B. juncea* lines. M- protein marker; C- control plant; Lanes 1–10- T_0 lines (**a**), Lanes 1– 4 T_1 lines (**b**). Marker lane was cut and overlapped with the zymogram

reduction in lesion area as compared to control plants, and a negative correlation between endochitinase activity and % lesion area (R = − 0.91) (Fig. 7c). When challenged with *A. brassicae*, T_0 lines showed 30.9–62 % reduction in lesion area with negative correlation between endochitinase activity and lesion size (R = − 0.96) (Fig. 7b) while T_1 lines showed 30.5–73 % reduction in lesion area with a negative correlation (R = − 0.91) between endochitinase activity and lesion size (Fig. 7d).

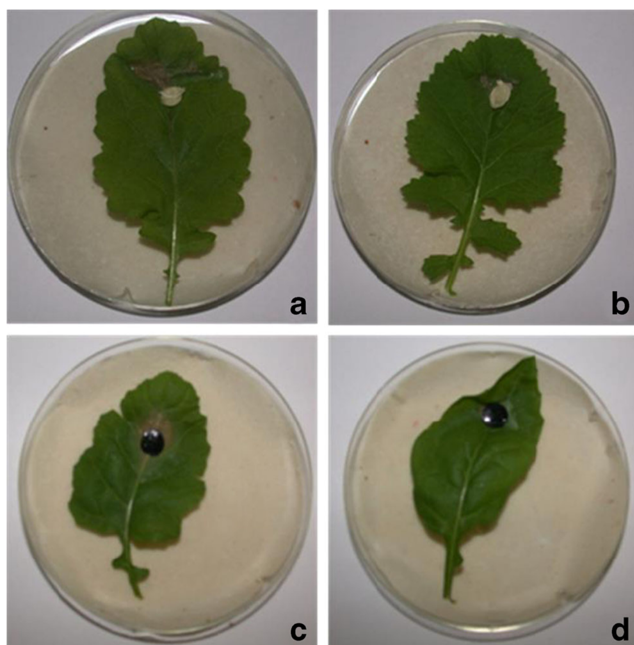

Fig. 6 A representative photograph of transgenic *B. juncea* lines showing resistance to two fungi, *Alternaria brassicicola* (**a**-nontransgenic plant; **b**-transgenic plant) and *Alternaria brassicae* (**c**-nontransgenic plant; **d**- transgenic plant) in detached leaf bioassay

Discussion

In the present study, we have developed transgenic *B. juncea* lines expressing an endochitinase '*ech42*' gene from *T. virens*. In a detached leaf bioassay, these lines showed enhanced tolerance against two fungi, *A. brassicicola* and *A. brassicae*, major pathogens of *Brassica* species. Integration, expression and copy number of transgenes in the transgenic lines (T_0 and T_1) were confirmed by PCR, RT-PCR and Southern blot analysis respectively. In Southern blot, all but one T_0 lines showed the presence of a single copy of the transgene. This confirms stable integration of '*ech42*'gene in the plant genome. Though line 2 showed 2 copies of '*ech42*' gene, the plant did not show any significant difference in endochitinase activity as well as fungal resistance compared to other transgenic lines. An enhancement in endochitinase activity to the tune showed 1.5 to 6.9 fold in T_0 lines and 4.2 to 6.7 fold in T_1 lines. The variation in the endochitinase activity in different transgenic lines could be due to the varied level of activity of 35S promoter which is known to be influenced by several regulatory controls (Benefy and Chua 1990; Emani et al. 2003). In the present study, some transgenic lines of *B. juncea* showed different leaf morphology (reduction in leaf size) and stunted growth which is known in apple expressing '*ech42*' gene from *Trichoderma atroviride* (Bolar et al. 2000).

In detached leaf bioassay, transgenic *B. juncea* lines (T_0 and T_1) showed moderate reduction in disease severity, even though the endochitinase activity level in the plants was higher compared to non-transgenic plants. Similar results have been reported by Mora and Earle (2001) in transgenic broccoli plants with '*ech42*' gene from *T. harzianum* when challenged with *A. brassicicola*. Mondal et al. (2003) reported overexpression of 'glucanase' gene in transgenic *B. juncea*

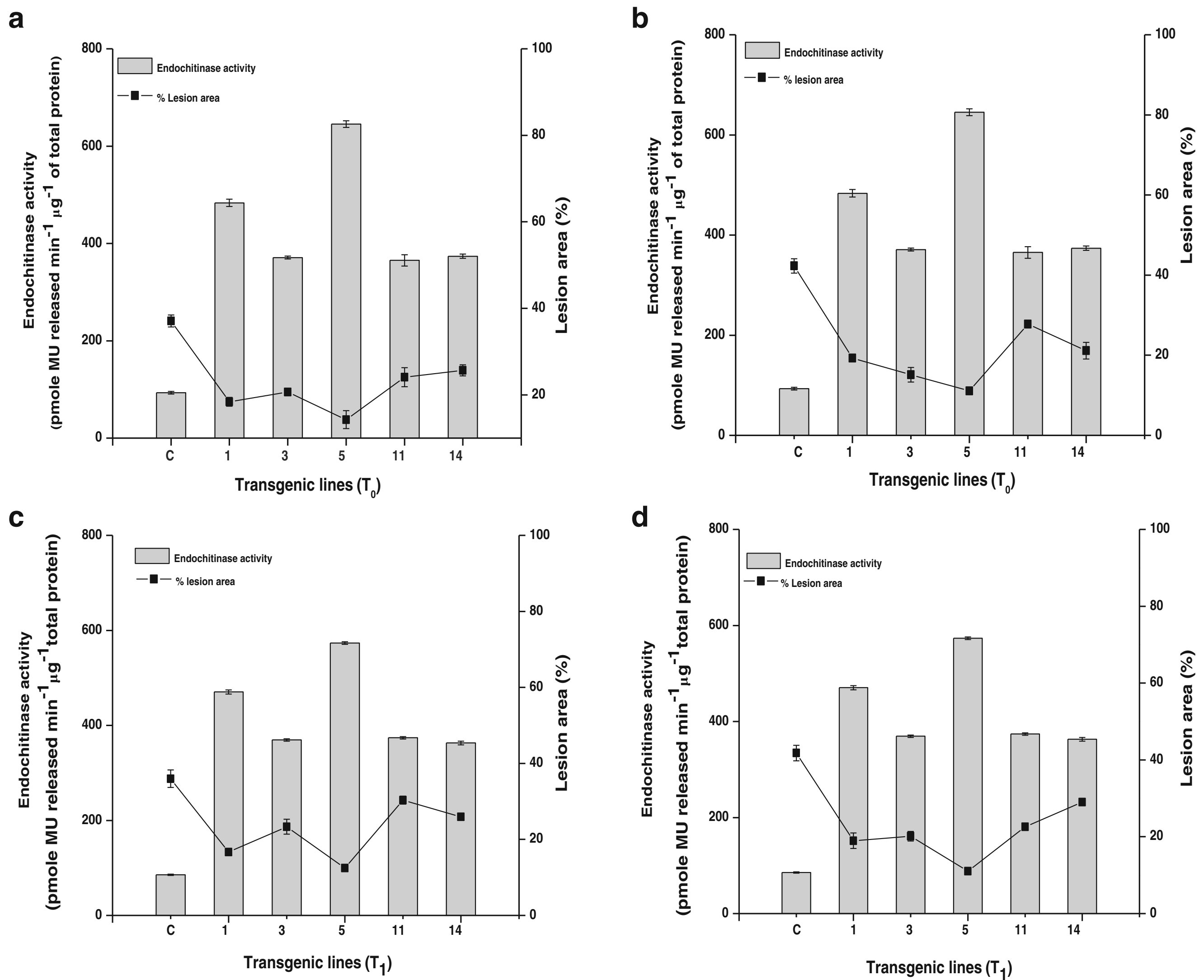

Fig. 7 Endochitinase activity and average lesion area in control and transgenic *B. juncea* lines. (**a** and **b**: T_0 lines challenged with *A. brassicicola* and *A. brassicae* respectively); (**c** and **d**: T_1 lines challenged with *A. brassicicola* and *A. brassicae* respectively). Error bars indicates ± standard error of means. The values for the all the transgenic lines are significantly different from the control plant at $p < 0.01$

lines showing a 10–15 days delay in onset of fungal infection. Overexpression of '*ech42*' gene from *T. virens* in transgenic tobacco and tomato plants also showed strong negative correlation between endochitinase activity and percentage leaf area infected with two fungi, *B. cinerea* and *A. alternata* respectively (Shah et al. 2010). In the present study, leaves of transgenic *B. juncea* lines showed 4–5 day delay in the onset of fungal infection while leaves of control plant showed lesions within 2 days.

Whether or not plants transformed with chitinase genes are protected against fungal pathogens appears to depend on multiple factors, including the source of the transgene, the crop transformed, the pathogen tested, and the rigor of the statistical analysis applied (Mora and Earle 2001). In the present study, the range of endochitinase activity in T_1 generation provided similar levels of protection as in T_0 *B. juncea* lines against *A. brassicae* and *A. brassicicola* confirming stable expression of '*ech42*' gene in subsequent generations and the transgenic *B. juncea* lines were found to be equally tolerant to both *A. brassicae* and *A. brassicicola*. Considering the importance of *B. juncea* as an important oil seed crop in the Indian subcontinent and its yield losses due to fungal diseases, an endochitinase gene '*ech42*' from *T. virens* could be a potential candidate gene for development of disease resistant *B. juncea* plants in combination with other disease resistant genes.

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