CURRENT MICROBIOLOGY Vol. 47 (2003), pp. 171–173
DOI: 10.1007/s00284-002-3980-6

Current Microbiology
An International Journal
© Springer-Verlag New York Inc. 2003

Chitinase-Mediated Inhibitory Activity of Brassica Transgenic on Growth of *Alternaria brassicae*

Kalyan K. Mondal,[1] Subhas Chandra Chatterjee,[2] Navin Viswakarma,[3] Ram Charan Bhattacharya,[3] Anita Grover[3]

[1]Choudhary Swaran Kumar Himachal Pradesh Agricultural University, Research Station, Sangla Valley, Kuppa (Zira Farm), Kinnaur-172 106, Himachal Pradesh, India
[2]Division of Plant Pathology, Indian Agricultural Research Institute, Pusa Campus, New Delhi 110 012, India
[3]National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, Pusa Campus, New Delhi 110 012, India

Received: 23 September 2002 / Accepted: 24 October 2002

Abstract. Chitinase, capable of degrading the cell walls of invading phytopathogenic fungi, plays an important role in plant defense response, particularly when this enzyme is overexpressed through genetic engineering. In the present study, Brassica plant (*Brassica juncea* L.) was transformed with chitinase gene tagged with an overexpressing promoter 35 S CaMV. The putative transgenics were assayed for their inhibitory activity against *Alternaria brassicae*, the inducer of Alternaria leaf spot of Brassica both in vitro and under polyhouse conditions. In in vitro fungal growth inhibition assays, chitinase inhibited the fungal colony size by 12–56% over the non-trangenic control. The bioassay under artificial epiphytotic conditions revealed the delay in the onset of disease as well as reduced lesion number and size in 35S-chitinase Brassica as compared to the untransformed control plants.

Alternaria blight caused by *Alternaria brassicae* is a serious threat to mustard (*Brassica juncea*) cultivation [16]. The expensive and non-ecofriendly conventional chemical approach is practically insufficient to combat the new emerging virulent pathotypes [4, 5]. Further, the development of resistant lines/cultivars through breeding approach is of limited scope due to the lack of suitable resistant donors [4]. As an alternative, the constitutive expression of pathogenesis-related proteins (viz. chitinase, glucanase, osmotin, etc.) in the plant system through genetic engineering approach has got prime importance [4, 12]. Chitinase catalyzes the hydrolysis of chitin, a β-1-4-linked polymer of N-acetyl D-glucosamine and a major component of the cell wall of most filamentous fungi [2, 3, 7, 9, 10]. Chitinase overexpression in transgenic plants leading to increased resistance to fungal pathogens has been reported [2, 3, 7, 9, 10, 15]. Effort in this direction in Indian Brassica has also been employed [3, 4, 5, 12]. However, the conformation of transgene expression in the transformed plants through molecular analysis would in no way be effective in inducing defense response in the targeted plants until their performance is judged through pathogen challenged conditions. In the present study, the performance of the chitinase transgenic of Indian Brassica under pathogen challenged conditions (both in vitro and polyhouse conditions) are discussed.

Correspondence to: K.K. Mondal; *email:* kalyanmondal@yahoo.com

Materials and Methods

The gene construct of chitinase designed for overexpression and suitable for plant transformation was used to transform *Brassica juncea* (cv.RLM 198) [18]. Five independent transgenic lines were confirmed for gene integration [6, 7] by PCR and Southern hybridization techniques [14].

In vitro inhibitory assay. The protein chitinase extracted from leaf of transgenic *Brassica juncea* was used for in vitro inhibitory effect on growth of *Alternaria brassicae*, the incitant of Alternaria blight of rapeseed-mustard. The protein from non-transgenic plants and protein extracting buffer were served as control. Five wells (4 mm diameter) were made on antibiotic assay agar plate (AAA, Himedia Co.) and wells were first filled with 50 μl of spore suspension (2×10^6 spores/ml). After keeping overnight at 30°C in BOD incubator, each well was treated with 50 μl of either leaf extract, boiled transgenic leaf extract, buffer, or commercial chitinase (1.5 μg/40 μl). The observations were recorded as radial growth of *A. brassicae* at 25°C after 24 h intervals up to 5 d. The percent inhibition of hyphal growth was calculated based on the following formula:

$$\text{Percent inhibition} = \frac{\text{diameter of fungal colony in treatments}}{\text{diameter of fungal colony in control (untransformed plant)}} \times 100. \quad (1)$$

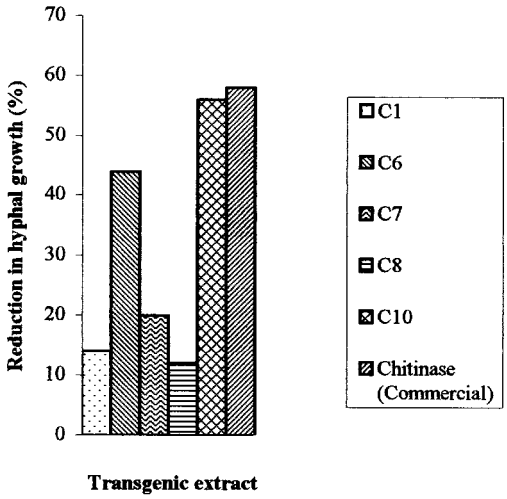

Fig. 1. Percent reduction in hyphal growth of *A. brassicae* by transgenic leaf extracts.

Polyhouse screening of transgenic. The transgenics were subjected to artificial epiphytotic conditions maintained in a polyhouse. The temperature was maintained around 26°C and humidity was around 80%. Fifty plants of each independent transgenic clone were assayed. The spore suspension (1×10^6 spores per ml) in water was prepared from virulent isolate of *Alternaria brassicae* (72-h-old culture). The plants were kept turgid by spraying sterile water and covered with a polypropylene bag before 8 h of inoculation. Then plants were inoculated with spore suspension by atomizing. The untransformed plant was maintained as control. The observations were recorded as number of lesions per leaf (average of three leaves per plant) and lesion diameter (in mm) after 5 d of inoculation. Percent disease protection was calculated as the number of lesions per leaf in transgenic divided by the number of lesions per leaf in untransformed plant, multiplied by 100.

Results and Discussion

In the present study, five independent clones of 35S-chitinase Brassica (*Brassica juncea* L.) cv. RLM 198 were assayed for their efficacy in inhibiting the growth of *Alternaria brassicae* (the incitant of Alternaria leaf spot of Brassica) both under in vitro as well as under polyhouse conditions. The extract from 35S-chitinase Brassica reduced the hyphal growth by 12–56 percent over the untransformed control (Fig. 1). Among the five independent clones of 35S-chitinase Brassica, clone C_{10} performed best in inhibiting hyphal growth (by 56%), which is almost closer to that of commercial chitinase (Fig. 2), followed by the clone C_6 (44%) under in vitro fungal growth inhibition assays (Fig. 1). No inhibition was detected in the presence of either boiled extract or extracting buffer (Fig. 2) indicating that the observed growth inhibitory effect may arise from enzyme-catalyzed hydrolysis of newly formed chitin and resultant disruption of the growing hyphal tips [3, 11, 13, 17]. Studies at protein level also showed two- to fourfold

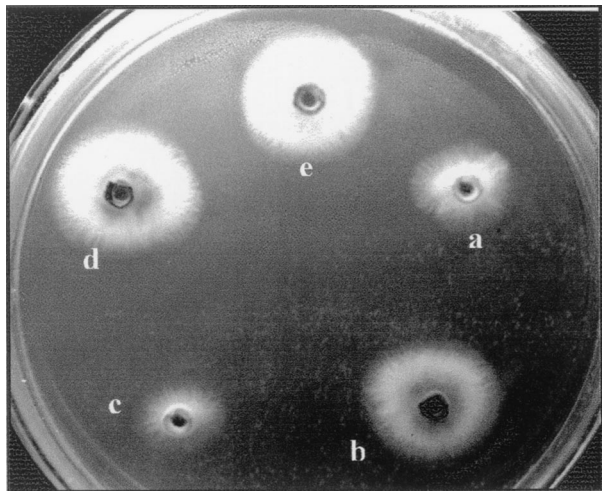

Fig. 2. In vitro growth inhibition activity of leaf extracts obtained from 35S-chitinase Brassica (C_{10} clone) after 24 h of incubation. Each well [containing 50 μl spore suspension (2×10^6 spores/ml)] treated with 50 μl of either transgenic extract (a), boiled transgenic extract (b), commercial chitinase at 1.5 μg/40 μl (c), non-transgenic extract (d), or buffer used for leaf extraction (e).

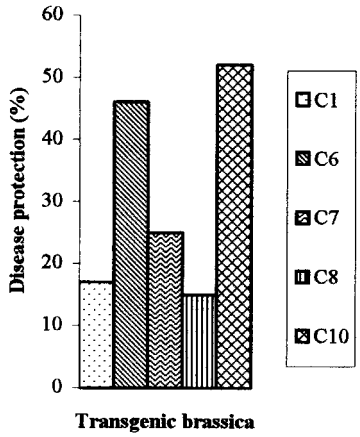

Fig. 3. Performance of 35S-chitinase Brassica in protecting the Alternaria leaf spot over control (untransformed) plants under polyhouse conditions.

increases in enzyme expression in transgenic plants compared to non-transgenic plants [6].

The polyhouse screening assay of transgenic revealed that the clones C_{10} and C_6 exhibited quite appreciable suppression of the disease (52 and 46%, respectively) over the non-transgenic control (Fig. 3). The lesions on leaves of the transformed Brassica were found to be reduced in diameter (2–4 mm in diameter) as well as in number (less than 10 lesions per leaf) as compared to the untransformed Brassica (5–15 mm in diameter and more than 50 lesions per leaf) under artificial epiphytotic conditions. The overexpression of chitinase in transgenic

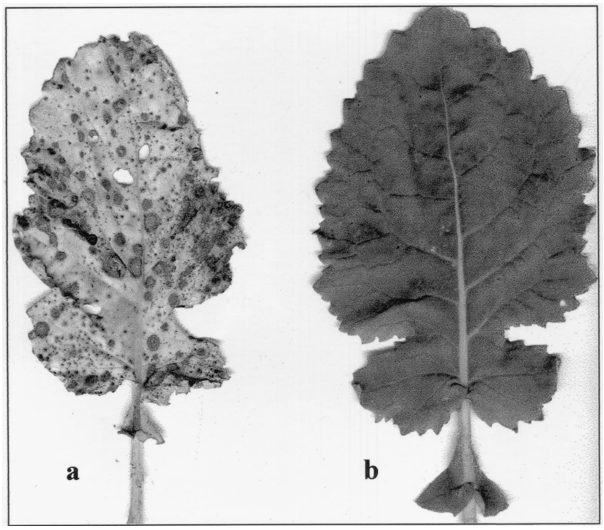

Fig. 4. Leaves challenged with *A. brassicae* showing minute lesions in the case of 35S-chitinase Brassica (b) as compared to the untransformed control (a) under polyhouse conditions.

plants leading to increased resistance to fungal pathogens have been well documented in different host-pathogen systems [1, 3, 6, 8, 9, 10, 12, 19]. Further, the onset of disease (as indicated by the appearance of lesions) was delayed by a period of 10–15 d in the transgenic Brassica as compared to the untransformed control plants. This in turn suggested that the pathogen took longer to get established into the host harbouring chitinase genes, resulting in a delay in disease inception as well as reduced lesion size [3]. The delay in appearance of symptoms as well as the lower severity of disease was observed when 35S-chitinase tobacco was challenged to *Rhizoctonia solani* (causing root rot disease) infection [3]. Therefore, the present study concluded with the fact that the overexpression of chitinase gene through 35S promoter in Brassica resulted in activation defense response against the fungal pathogen *Alternaria brassicae* and thereby protecting the plant in terms of decreased severity as compared to the untransformed control.

Literature Cited

1. Bliffeld M, Mundy J, Potrykus I, Futterer J (1999) Genetic engineering of wheat for increased resistance to powdery mildew disease. Theor Appl Genet 98:1079–1086
2. Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. Ann Rev Phytopathol 28:133–138
3. Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Trangenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254: 1194–1197
4. Chatterjee SC, Mondal KK (1999) Disease management approaches for rapeseed-mustard: to meet the challenges for next millennium. In: Proceeding of National Symposium on Crop Pest and Disease Management: Challenges for the next millennium. New Delhi: Society of Plant Protection Sciences, pp 9–10
5. Chatterjee SC, Mondal KK (2000) Inhibitory effect of transgenic *Brassica juncea* over expressing chitinase gene on growth of *Alternaria brassicae*. In: Proceeding of National Symposium on Role of Resistance in Intensive Agriculture. New Delhi: Indian Council of Agricultural Research, pp 50–51
6. Grover A, Arora N, Mondal KK, Sen A, Chatterjee SC, Kirti PB, Bhat SR, Sharma RP (2001) Development of transgenic *Brassica juncea* plants resistant to fungal pathogens using chitinase and glucanase genes. In: Proceedings of 88th Indian Science Congress, part III. New Delhi, pp 13–14
7. Grover A, Yasphal, Sen A, Arora N, Kriti PB, Sharma RP (2001) Removal of vacuolar targeting signal from class 1 vacuolar chitinase leads to its extracellular secretion in transgenic tobacco. J Plant Biochem Biotechnol 10:139–142
8. Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Mass C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 8:97–109
9. Jongedijk E, Tigelaar H, van Roekel JSC, Bres Vloemans SA, Dekker I, van den Elzen PJM, Cornelissen BJC, Melchers LS (1995) Synergistic activity of chitinases and ~1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173–180
10. Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. Biotechnology 13:686–691
11. Mauch F, Mauch-Mani B, Boller J (1988) Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and beta-1,3-glucanase. Plant Physiol 88:936–942
12. Nishizawa Y, Nishio Z, Nakazono K, Soma M, Nakajima E, Ugaki M, Hibi T (1999) Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic rice by constitutive expression of rice chitinase. Theor Appl Genet 99:383–390
13. Ponstein AS, Bress-Vloemans SA, Sela-Buurlage MB, Van de Elzen PJM, Melchers LS Cornelissen BJC (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol 104:109–118
14. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, second edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press
15. Sela-Buurlage MB, Ponstein AS, Bresvloemans SA, Melchers LS, Van de Elzen PJM, Cornelissen BJC (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and 1,3-glucanases exhibit antifungal activity. Plant Physiol 101:857–863
16. Singh RS (1990) Plant diseases, sixth edn. New Delhi: Oxford and IBH
17. Woloshuk CP, Meulenhoff JS, Sela-Buurlage MB, Van de Elzen PJM, Cornelissen BJC (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3:619–628
18. Yasphal, Sen A, Venkateshewari J, Kriti PB, Sharma RP, Grover A (1999) Pyramiding chitinase and glucanase genes for fungal resistance. Indian J Experiment Biol 37:579–583
19. Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Biotechnology 12:807–812