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Heterologous expression of new antifungal chitinase from wheat

Arpita Singh, S. Isaac Kirubakaran, N. Sakthivel *

Department of Biotechnology, School of Life Sciences, Pondicherry University, Kalapet, Puducherry 605 014, India

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Abstract

Chitinases (EC 3.2.1.14) have been grouped into seven classes (class I–VII) on the basis of their structural properties. Chitinases expressed during plant–microbe interaction are involved in defense responses of host plant against pathogens. In the present investigation, chitinase gene from wheat has been subcloned and overexpressed in *Escherichia coli* BL-21 (DE3). Molecular phylogeny analyses of wheat chitinase indicated that it belongs to an acidic form of class VII chitinase (glycosyl hydrolase family 19) and shows 77% identity with other wheat chitinase of class IV and low level identity to other plant chitinases. The three-dimensional structural model of wheat chitinase showed the presence of 10 α-helices, 3 β-strands, 21 loop turns and the presence of 6 cysteine residues that are responsible for the formation of 3 disulphide bridges. The active site residues (Glu94 and Glu103) may be suggested for its antifungal activity. Expression of chitinase (33 kDa) was confirmed by SDS–PAGE and Western hybridization analyses. The yield of purified chitinase was 20 mg/L with chitinase activity of 1.9 U/mg. Purified chitinase exerted a broad-spectrum antifungal activity against *Colletotrichum falcatum* (red rot of sugarcane) *Pestalotia theae* (leaf spot of tea), *Rhizoctonia solani* (sheath blight of rice), *Sarocladium oryzae* (sheath rot of rice) *Alternaria* sp. (grain discoloration of rice) and *Fusarium* sp. (scab of rye). Due to its innate antifungal potential wheat chitinase can be used to enhance fungal-resistance in crop plants.

Keywords: Wheat chitinase; Antifungal protein; Molecular phylogeny; *Escherichia coli* expression

Plants do not contain an immune system and thus are vulnerable to pathogens, resulting in significant crop loss globally [42]. In order to protect themselves from pathogens, plants have evolved a number of defense responses that are elicited during their life cycle in response to developmental signals and pathogen attack. Plants express a wide variety of pathogenesis-related (PR) protein encoding genes [6] of which the best characterized are those encode the lytic enzyme chitinase (EC 3.2.1.14). Chitinases are speculated to play a vital role in plant defense mechanisms against fungal pathogens due to its ability to digest chitin, a major constituent of the cell wall of a number of phytopathogenic fungi [3,4,34]. Chitinases hydrolyze β-1,4-glycoside bonds in chitin to produce sole reaction products *N*-acetylglucosamine and N,N^{+}-diacetylchitibiose [10], also involved in the chitin breakdown of soil microbes, in the molting process of insects and in defense against chitin-containing phytopathogenic fungal infection [47]. The acidic and basic isoforms of chitinases are induced in plants in response to pathogen attack, other environmental stimuli and are also expressed in certain tissues of plants during normal development [3,4,6,34]. Chitinases isolated from both monocot and dicot plants have been shown to inhibit the growth of fungi *in vitro* [31,43,47,59]. Chitinase isozymes have been divided into seven classes (class I–VII) on the basis of their structural properties [40] and further divided into family 18 and 19 of glycoside hydrolase based on primary sequence and catalytic mechanisms (Carbohydrate Active Enzyme (CAZy)[1] database; URL: http://afmb.cnrs-mrs.fr/CAZY/). The catalytic domains in family

* Corresponding author. Fax: +91 413 2655265, +91 413 2655179.
E-mail address: puns2005@gmail.com (N. Sakthivel).

[1] *Abbreviations used:* CAZy, carbohydrate active enzyme; LB, Luria–Bertani; NJ, neighbour-joining; CBD, chitin binding domain.

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18 chitinases have a $(\beta/\alpha)_8$ where as family 19 chitinases have high α-helical contents and share some structural similarity with chitosanases and lysozyme. Family 18 chitinases use substrate assisted double-displacement mechanism whereas family 19 chitinase use a single-displacement mechanism [14]. Class I chitinases are basic and contain a cysteine-rich N-terminal domain with putative chitin binding properties. They are usually localized in the vacuole and are potent growth inhibitors of many fungi [26,37]. Class II chitinases are generally acidic and extracellular, and can be detected in the apoplastic fluid or culture medium of protoplasts [2,16]. A possible role for these chitinases in plant defense is to act as signaling molecules, releasing elicitors from invading fungal hyphae and acting as a first line of defense [37]. Class III chitinases are extracellular hydrolases whose conserved catalytic-domain amino acid sequence differs from the conserved sequence of class I or II chitinases. Most of the class III chitinases are classified on the basis of their identity to previously described lysozymes with chitinase activity [18]. A newly proposed group of class IV chitinases that lacks C-terminal extension are assumed to be accumulated extracellularly [11,39]. PR-3 chitinases include class Ia, Ib, II, IV, V, VI and VII chitinases. PR-8 and PR-11 chitinases include chitinases of class III and I, respectively [1,38].

Biotechnological approaches for crop protection through engineered genetic disease resistance is emerging as one among the promising tools that eliminates the risks and disadvantages associated with traditional methods. Transgenic plants overexpressing PR proteins have been reported to demonstrate the enhanced resistance to fungal diseases [12,49]. The prerequisite for obtaining promising transgenic plants resistant to fungal pathogens is the isolation and characterization of genes encoding broad-spectrum antifungal protein from different plant species. By screening the wheat genomic library, chitinase clone was isolated [32]. In a recent study, antifungal potential of class I chitinase from barley was reported [22]. The purpose of present investigation was to isolate and overexpress the wheat chitinase gene and to evaluate its antifungal activity against major phytopathogenic fungi that attack economically important agricultural crop plants.

Materials and methods

Bacterial strains, plasmid and fungal cultures

The host bacterium *Escherichia coli* strain BL-21 (DE3) and the expression vector pET 28a+ (Novagen Madison, WI, USA) were used in this study. PCR reagents, T4 DNA ligase and restriction endonuclease were purchased from Promega, Madison, WI, USA. *E. coli* cells with plasmids were grown aerobically in Luria–Bertani (LB) broth (Hi-Media Laboratories, Mumbai, Maharastra, India) or on (LB) agar plate, supplemented with kanamycin (50 μg/ml) for the selection of transformants. Wheat chitinase cDNA clone (SM194) was kindly supplied by Dr. S. Muthukrishnan, Department of Biochemistry, Kansas State University, USA. Phytopathogenic fungal species used in this study include *Alternaria* sp. (grain discoloration in rice), *Bipolaris oryzae* (brown spot of rice), *Magnaporthe grisea* (blast of rice), *Sarocladium oryzae* (sheath rot of rice), *Rhizoctonia solani* (sheath blight of rice), *Macrophomina phaseolina* (charcoal rot of groundnut), *Botrytis cinerea* (blight of tobacco), *Pestalotia theae* (leaf spot of tea), *Curvularia lunata* (leaf spot of clover), *Colletotrichum falcatum* (red rot of sugarcane), *Colletotrichum gleosporoides* (anthracnose of mango), *Fusarium* sp. (scab of rye), *Cylindrocladium scoparium* (root necrosis of banana) and *Cylindrocladium floridanum* (root necrosis of banana). Rice, groundnut, tobacco, tea, clover sugarcane and mango infecting fungi were isolated by Dr. N. Sakthivel, Pondicherry University, India and the banana infecting fungi were isolated by Dr. J. M. Risede, UMR de pathologie Vegetale INRA-INH-Universite d'Angers, Beaucouze Cedex and CIRAD-FLHOR, France. All fungi were maintained at Microbial Culture Collection (MCC), Department of Biotechnology, Pondicherry University, Puducherry, India.

Nucleic acid techniques

Plasmid isolation, plasmid transformation and standard DNA protocols were performed as described Sambrook et al. [45].

DNA sequencing, editing and molecular phylogeny analyses

Wheat cDNA clone SM194 was sequenced using the facility available at Macrogen Inc. (Seoul, Korea), edited and analyzed using Clone Map version 2.11 (CGC Scientific Inc., MO, USA). The reference sequences required for comparison were downloaded from the National Centre of Biotechnology Information (NCBI). The software ClustalW (1.82) from Europe Biotechnology Information (EBI) (www.ebi.ac.uk) was employed for multiple sequence alignment of amino acid sequences of wheat chitinase with other plant chitinases retrieved from GenBank and subsequently, the phylogenetic tree was constructed by neighbour-joining (NJ) method [44] with ClustalX1.81 [57]. The reliability of the tree was measured by bootstrap analysis with 1500 trials and the phylogenetic tree was edited using molecular evolutionary genetics analysis and sequence alignment tool MEGA v 3.1 [28].

Molecular modeling

Barley chitinase (Protein Data Bank code 1CNS) was used as the template. Modeling was done using molecular operating environment (MOE version 2001.07) [9]. Ten intermediate homology models were built as a result of the permutational selection of different loop candidates and side chain rotamers. The intermediate models were averaged to produce the final model by Cartesian average.