Overexpression of rice *chitinase* gene: Evaluation of chitinase ability as a bio-antifungal agent

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ABSTRACT

Seven local fungal isolates of Pyricularia grisea were purified from infected rice leaves. The total proteins were extracted and SDS-PAGE was carried out to differentiate between the expression of proteins in infected and healthy plants. SDS-PAGE analysis revealed the accumulation of ~35-kDa chitinase after 16, 20, 24 and 48 hr (hours post inoculation). Rice chitinase gene (1.023 bp) was successfully amplified from the total RNA extracted from infected rice using RT-PCR. The amplified fragment was cloned and overexpressed in E. coli BL21 cells as 6x-His- fusion protein. Recombinant chitinase fusion protein was successfully purified using Ni-NTA affinity column chromatography. Two chitinase activity assays against P. grisea were carried by the filter disc and the dissimilar concentrations plates method. The results indicated that the expressed chitinase protein had an antifungal activity against P. grisea.

Key words: Rice chitinase gene, anti-fungal agent.

INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple food crop for half of the world's population. Rice blast disease is a serious agricultural problem worldwide (Ou, 1985). Outbreaks of rice blast disease are a serious and recurrent problem in all rice-growing regions of the world, and the disease is extremely difficult to control (Valent and Chumley, 1991; Talbot, 2003). Rice blast, caused by the fungus *Magnaporthe grisea*, is therefore a significant economic and humanitarian problem. It is estimated that each year enough rice is destroyed by rice blast disease to feed 60 million people. *M. grisea* is the most destructive pathogen of rice worldwide; around 50% of the production may be lost in a field moderately affected by infection (Zeigler *et al*., 1994). The disease is currently managed using resistant cultivars, fungicides and cultural practices. Most of the rice cultivars are susceptible to some strains of this fungus and since the pathogen is highly variable and breeding for durable resistance to blast remains a major challenge (Roy-Barman and Chattoo, 2005). Fungicides are commonly used to control blast. However, these are becoming less acceptable as they increase the potential for build-up of resistance in *M. grisea* to fungicides and also conflict with the public concern for fungicide residues on human health and environment (Coca *et al*., 2006).

Plants protect themselves against pathogen attack by launching both localized and systemic defense responses such as the generation of reactive oxygen species, the accumulation of phytoalexins, and the synthesis of pathogenesis-related proteins (PR) such as chitinases, β-1,3-glucanases, and thaumatin-like proteins (Bowles, 1990). The best characterized genes belonging to this group are those that encode the lytic enzyme chitinase, which is speculated to play a vital role in plant defense against fungal pathogens (Kirubakaran and Sakthivel, 2007). Different genetic strategies have been used to generate anti-fungal proteins which include utilization of antimicrobial genes of both plant and non-plant origin (Cao *et al*., 1998; Lorito *et al*., 1998; Datta *et al*., 1999). The best known examples of protection conferred by transgenic expression of plant antifungal genes are represented by overexpression of chitinases and b-1, 3-glucanases (Broglie *et al*., 1991; Datta *et al*., 1999).

The main goals of this study were to purify the fungal isolates that infect the Egyptian rice cultivars by blast disease, identification and characterization of the gene-enzyme system of chitinase and finally, the overexpression in *E. coli* expression system and evaluate its ability as a bio-antifungal potential against major phytopathogenic fungi that attack rice.

MATERIALS AND METHODS

Plant material

One cultivar of *Oryza sativa* (Sakha 104) was used in this study as a host plant for the artificial infection with *P. grisea* isolates as a source of *chitinase* gene. The cultivar was obtained from Field Crops Research Institute, Agricultural Research Center, Giza, Egypt.

Fungal isolates and plasmids

Seven local fungal isolates of *P. grisea* were collected from different regions (Dakahliyah, Beheira, Desouk, Qutur, Mahalla, Sakha and Basyon) in Egypt. The isolates were purified from infected rice leaves having the fungal infection symptoms. The isolates were grown in banana dextrose agar (BDA) medium at 28 °C.

PBAD-TOPO TA Expression Kit (Invitrogen, USA) was used for the direct insertion of *Taq* polymerase amplified PCR products into a plasmid vector for regulated expression in *E. coli* strain BL21. All steps were performed as described in the manufacturer's instructions.

Artificial infection

The fungal isolates were used to inoculate rice plants to identify the more virulent isolates in infection as described by Radjacommare *et al.* (2004). Also**,** artificial infection was done to determine the induced rice blast proteins upon infection with *P. grisea* after 16, 20, 24, and 48 hr (hours post inoculation), special chitinase protein.

SDS- PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for total proteins was carried out to differentiate between the expression of proteins in infected and healthy plants using the method described by Laemmli (1970). Also, this technique was used to examine the expression of *chitinase* gene after constructing it into the expression vector.

Amplification of cDNA for *chitinase* gene

Frozen rice leaves infected with *P. grisea* were ground in liquid nitrogen with a mortar and pestle. Total RNAs were isolated from healthy and infected plants after different time intervals, i.e., 10, 16, 20, 24 and 48 hr using a High Pure RNA Tissue Extraction Kit (Roche,

Diagnostics) according to the manufacturer's instructions. The isolated RNA was used as a template for synthesizing cDNA to be amplified by PCR. Two sets of primers Chit (+) '5-TTGGATCCAGAGCGTTCGTGTTG-3' and Chit (-) '5-TTTGGATCCAAGCGAAGGGCCTCTGGC TGTA-3' designed by Kirubakaran and Sakthivel (2007) were synthesized (SIGMA, USA) as a forward and reverse primers, respectively. A modification method of Pappu *et al*. (1998) was used for synthesizing cDNA strand by using Retrotools Reverse Transcriptase (from Biotools, Biotechnological & Medical Laboratories, S.A Madrid, Spain). A volume of 7 µl from the total nucleic acid primed with 50 pm/µl of minus sense primer Chit (-) in a total volume of 20 µl and placed in a water bath at 70 ^{o}C for 5 min. The reaction contained 4 µl of 5x RT buffer (Biotools, Biotechnological & Medical Laboratories, S. A. Madrid, Spain), 1 µl of 10 mM dNTPs, 1 µl of enhancing buffer, and 1.5 µl of Retrotools Reverse Transcriptase enzyme. The reaction was performed at 70 ^{o}C for 45-60 min. For PCR, 50 pm/µl of each amplification primer, (forward primer) and (reverse primer), was used. Five µl of each cDNA reaction, and 5 U/µl of High Expand Fidelity DNA polymerase (Roche) were used in a 5x Standard DNA buffer containing 20 mM Tris HCl, pH 8.2, 10 mM KCl, 6 mM $(NH4)_2$ SO_4, 2 mM $MnCl_2$, 0.1% Triton X-l00 and l0 µg/ml of nuclease-free BSA. The amplification reaction was carried out in a total volume of 25 µl using PCR thermal cycler, UNOII from Biometra and using 0.2 ml micro Amp PCR tubes. Hard denaturation of the DNA was performed at 95 ^{o}C for 2 min followed by 35 cycles of amplification with denaturation at 94 ^{o}C for 30 sec, annealing at 52 ^{o}C for 45 sec, and extension at 72 ^{o}C for 1 min. A single tailing cycle of long extension at 72^{o}C for 7 min was carried out in order to ensure flush ends on the DNA molecules. Finally, the amplification reactions were hold at 4°C. The amplified DNA was electrophoresed on 1 % agarose gel and photographed using gel documentation system (Biometra).

Overexpression of the recombinant *chitinase* gene in *E. coli*

The amplified fragment of the *chitinase* gene was cloned into the pBAD-TOPO expression vector to generate the recombinant plasmid pBAD-RC. The complete nucleotide sequencing of the recombinant plasmid pBAD-RC was determined using automated DNA sequencing to confirm that the *chitinase* gene was cloned. The pBAD-RC expression vector provides a high level of expression in *E. coli* as a fused protein with 6x His-tag. The amplified gene is under the control of tightly regulated araBAD promoter (P_{BAD}) to allow induction with 0.002 % of the *L-arabinose* for 5-6 hr according to recommended method.

Purification of the His-tagged chitinase fusion protein

Ni-NTA His-Bind Resin kit (Novagen, USA) was used to purify the over expressed recombinant chitinase protein. This kit was used for rapid one-step purification of protein containing a His-Tag sequence by metal chelation chromatography. The His-Tag sequence binds to Ni^{2+} cations, which are immobilized on the Ni-NTA His-Bind Resin. All steps of protein purification were carried out according to the manufacturer's instructions.

Chitinase activity as a bio-antifungal

Chitinase activity as a bio-antifungal against *P. grisea* was investigated by a hyphal extension inhibition assay (Radjacommare *et al*., 2004). The assay was carried out in 9 cm diam. petri plates containing 15 ml BDA medium. A mycelial disc of *P. grisea* was

placed at the centre and four or two sterile filter paper discs (6 mm diam.) were placed on the agar surface 3 cm from the centre. The different concentrations of purified chitinase (1, 2.5, 4, 6, 8 and 12 mg/ml) and buffer were applied on the filter paper in the petri plate and incubated at room temperature (27 ± 2 ºC). All enzyme preparations were sterilized by filtration through nylon disc filters. After 24, 48 and 72 incubation hours, the growth of *P. grisea* was observed.

Also, the chitinase activity was estimated as described by Johnson and Curl (1972) with slight modifications. The assay was carried out in cylinder plates containing BDA medium and various concentrations of chitinase protein (8, 12, 16 and 20 mg/ml). The mycelium disc of the test fungi was inoculated in the middle of the petri plates. The plates were incubated at 27 ± 2 ºC for 72 hr.

Microscopic examination of chitinolytic activity

The fungal hyphae from the periphery of the zone of inhibition produced by the purified chitinase was scraped, stained and examined under a scanning light microscope (Leica, Japan) (X400) for any morphological changes. A similar observation was also made for fungal hyphae obtained from a normal fungal culture (Kirubakaran and Sakthivel, 2007).

RESULTS

In this study, seven local fungal isolates of *P. grisea* were purified from infected rice leaves, IA-79, IB-63, IH-1, IC-13, ID-7, IF-3 and IA-77. To identify the more virulent isolate in infection, the spore suspension was sprayed on the 21 day old rice plants. The symptoms development was observed after 7 days and graded 0–5 scale based on the lesion size. The results indicated that ID-7 is the highly virulent isolate among the different isolates of the Egyptian rice cultivars. Consequently, the aggressive *P. grisea* ID-7 isolate was used in the present study for the artificial infection.

SDS- PAGE electrophoresis

Samples of infected leaves were collected from inoculated rice plants by *P. grisea* ID-7, after 16, 20, 24, and 48 hr. The total proteins were extracted, and then SDS-PAGE for total protein was carried out to differentiate between the expressed proteins in infected and healthy plants. Also, this test was carried out to estimate the level of chitinase expression in both infected and healthy plants.

Bands with different molecular weights were detected in the healthy and infected plants (Fig. 1). The healthy exhibited different protein profiles with infected plants in some bands. SDS-PAGE analysis revealed the accumulation of ~35-kDa protein after 16, 20, 24, and 48 hr. The intensity of the induced bands increased with increasing the time of infection that indicates that this band may be the chitinase protein with the expected molecular weight (35 kDa). Moreover, the high expression of chitinase was observed after 24 hr. Consequently, the chitinase is considered one of the rice blast resistance proteins induced by infection with *P. grisea*.

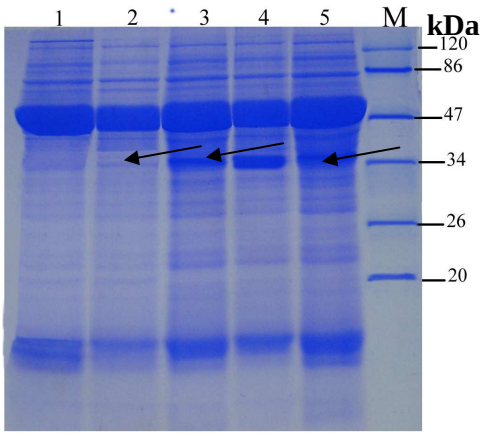

Fig. (1): SDS-PAGE analysis of total protein extracted from healthy and infected plants (treated with P. grisea). Lane 1, Healthy plant; Lanes 2, 3, 4 and 5, infected plants after 16, 20, 24 and 48 hr, respectively; M, wide range protein molecular weight marker (Fermentas). The arrows indicate the chitinase protein bands.

Identification of *chitinase* gene under fungal infection

Reverse transcription and PCR were carried out to amplify the coding sequence of rice *chitinase* gene using its specific primers. Samples of infected leaves with *P. grisea* ID-7, were collected after 10, 16, 20, 24 and 48 hr for total RNA isolation. In this procedure, reverse transcriptase enzyme was applied to synthesize the first cDNA from the total RNA by incubating the mixture at 70 ^{o}C for 45-60 min. PCR reaction was subjected to 35 cycles after initial denaturation for 5 min at 95°C. *Chitinase* gene (1.023 bp) was successfully amplified from the isolated RNA using the forward and reverse primers (Fig. 2). The results showed that a highly induced chitinase mRNA occurred after 24 hr, while the lowest induction was observed after 10 hr. The nucleotide sequencing and characterization of the amplified gene were carried out using automated DNA sequencer. The sequences confirmed that the amplified gene was the *chitinase* gene**.**

Cloning and overexpression of *chitinase* gene in *E. coli*

The cDNA of the *chitinase* gene was cloned into pBAD-TOPO expression vector. The vector was used for the direct insertion of the PCR fragment for regulating the expression in *E. coli*. The expression vector pBAD-TOPO was linear supplied with single 3´-thymidine (T) overhangs for TA cloning and topoisomerase (bound to the vector). *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The amplified fragment was cloned efficiently into pBAD-TOPO vector and transformed into an *E. coli* strain as described in the manufacturer's instructions. The Recombinant plasmid was termed pBAD-RC (Fig. 3). After transformation, screening of the positive colonies carrying the *chitinase* fragment were validated by PCR (Fig. 4). Recombinant plasmids were further analyzed by nucleotide sequencing to choose recombinant plasmids with *chitinase* gene in the correct orientation and in frame with 6xHis tag.

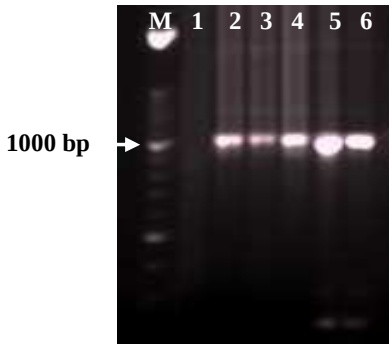

Fig. (2): Amplification of cDNA for the chitinase gene from infected plants after 10, 16, 20, 24 and 48 hr, respectively. (Lanes 2-6) show the expected molecular size of the chitinase gene (~1023 bp) compared to the non infected plant (Lane1) with no amplification results. M: DNA ladder (Roche).

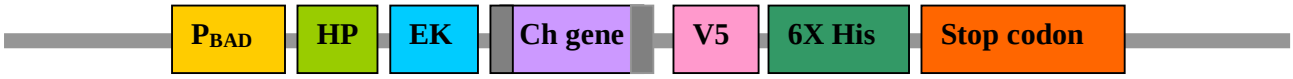

Fig. (3): pBAD-RC expression constructs.

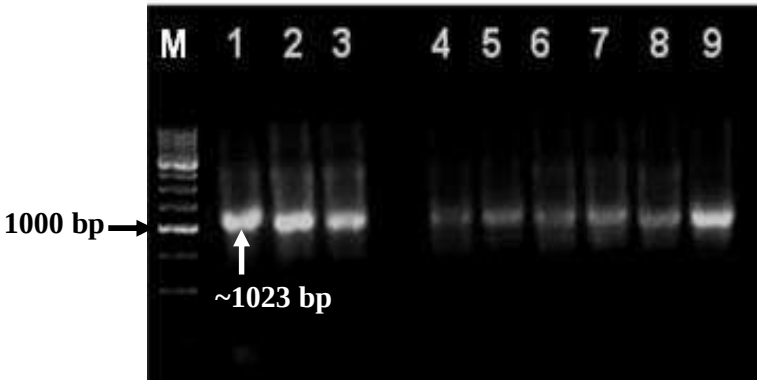

Fig. (4): Amplified chitinase cDNA (~1023 bp) from positive transformed clones (lanes 1 to 9); M: DNA ladder.

In pBAD-RC expression plasmid, the amplified gene was under the control of a tightly regulated P_{BAD} promoter to allow induction with L-arabinose. A single recombinant *E. coli* colony was grown to an OD_{600} = ~0.5 (to reach mid-log phase). The arabinose was added to the culture at 37 °C with shaking. The expression of the chitinase protein in the cell pellet was examined after 4 hr. SDS-PAGE technique was used to determine the success of expression experiments (Fig. 5; lanes 2-8). Results revealed that the expression of the ~35-kDa chitinase enzyme occurred with a high intensity band.

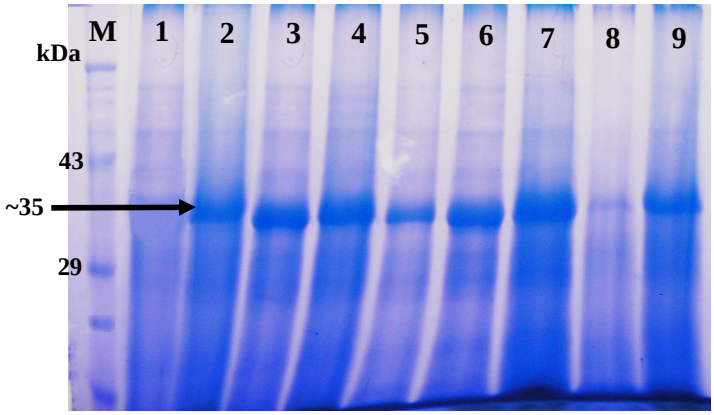

Fig. (5): SDS-PAGE analysis of chitinase protein. Lane 1, total protein pattern of E. coli lacking pBAD-RC (control); Lanes 2-8, total protein pattern of induced E.coli containing pBAD-RC; Lane 9, purified chitinase protein eluted using Ni-NTA column affinity chromatography. The arrow indicates the overexpressed chitinase protein (~35 kDa).

Purification of chitinase

Chitinase gene was successfully cloned in the pBAD-TOPO vector with an N-terminal hexahistidine tag and the chitinase enzyme was purified using Ni-NTA affinity column chromatography. With the His-Tag/His-Bind technology, purification is based on the affinity between the 6–10 neighboring histidines of the His-Tag sequence and an immobilized metal ion. The metal is held by chelation with reactive groups covalently attached to a solid support. The Ni-NTA His-Bind Resins use nitriloacetic acid (NTA) as the chelator, which has four sites available for interaction with metal ions. The clear supernatant containing soluble proteins was gently mixed with Ni–NTA resin for 2 hr and then loaded onto a 5 ml polypropylene plastic column. Contaminating proteins were washed from the column, and then the chitinase protein was eluted from the column. It is rarely necessary to remove the His-Tag sequence from the recombinant protein after purification. The purified protein was expressed at a level of about 20 mg/l of LB medium. The purity and molecular weight of the protein was estimated using SDS–PAGE (Fig. 5; lane 9).

Bio-antifungal activity of chitinase

Chitinase activity against *P. grisea* was carried out as described by Radjacommare *et al*. (2004) using filter paper discs. The fungal growth on plates was inhibited around the wells with chitinase added at concentrations of 6, 8 and 12 mg/ml for 48 and 72 hr (Fig. 6B, C, E and F). At 8 and 12 mg/ml concentrations for 48 and 72 hr, the growth was non-regular (Fig. 6E and F). Furthermore, the chitinase activity was estimated as described by Johnson and Curl (1972) using various concentrations of chitinase protein. The hyphal extension inhibition assay indicated that the fungal growth was lowest than control in all concentrations at all times (Fig. 7). The growth was lower at 16 and 20 mg/ml than the growth at 8 and 12 mg/ml. Moreover, the assay revealed that the growth was irregular and asymmetrical in 8 and 12 mg/ml for 72 hr. This result indicates that the chitinase protein had an antifungal activity against *P. grisea.*

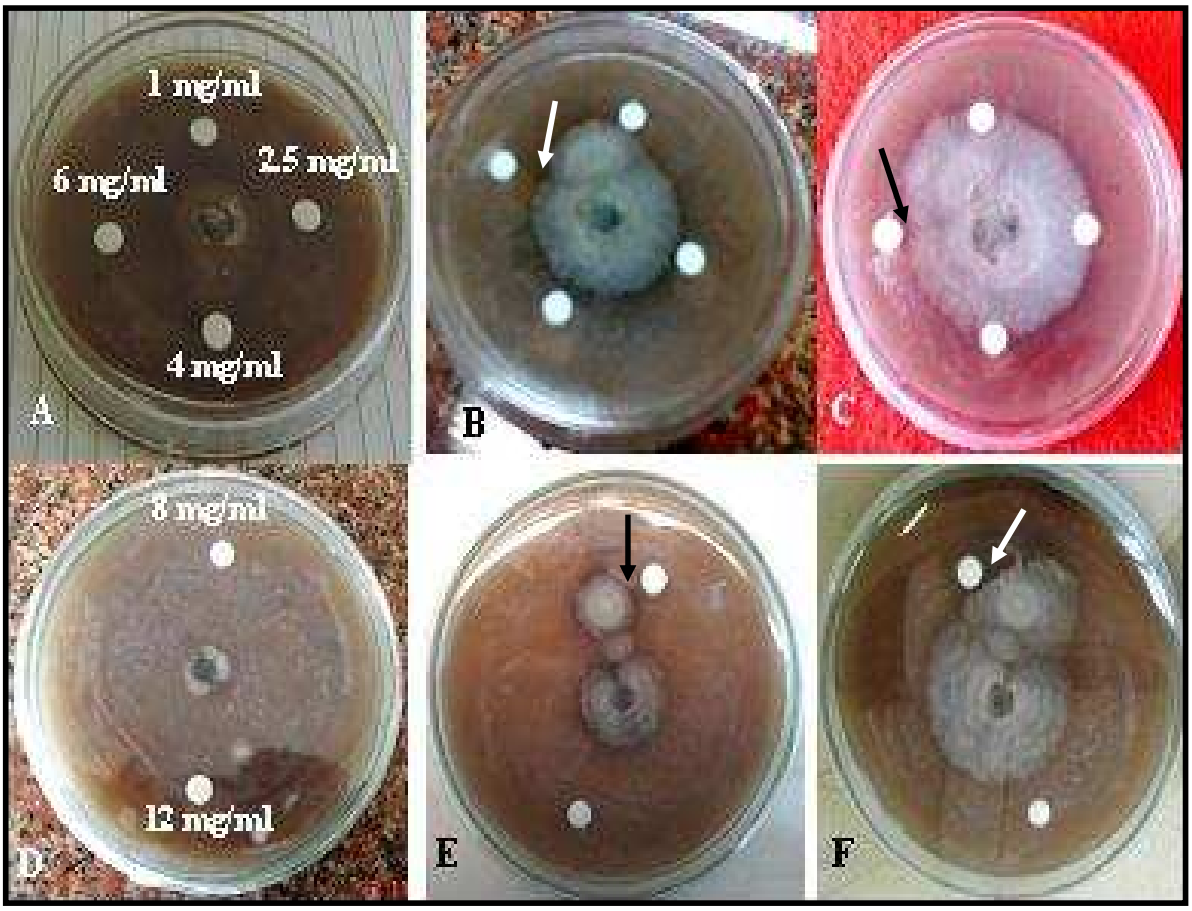

Fig. (6): Anti-fungal activity of the purified chitinase against P. grisea using filter discs. A and D after 24 hr; B and E after 48 hr; C and F after 72 hr. The arrows indicate the hyphal extension inhibition zones.

Microscopic analysis of chitinolytic activity

The formation of fungal hyphae was examined by scanning light microscope (SLM). The SLM image from the zone of inhibition plate of *P. grisea* revealed deformation (lysis and fragmentation) of the fungal mycelium and inhibition of mycelial branching (Fig. 8B), whereas the control showed highly branched and well developed mycelium (Fig. 8A).

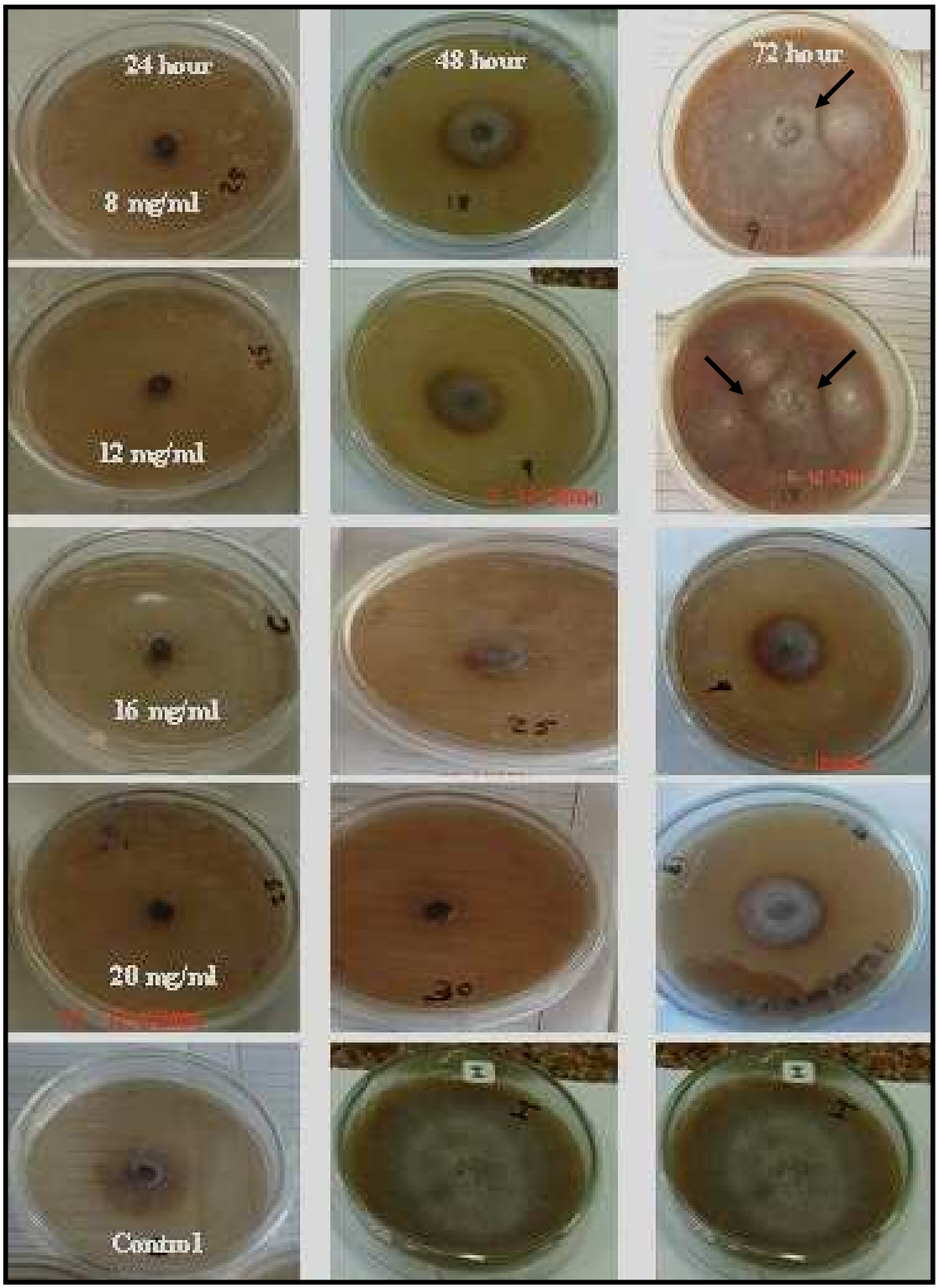

Fig. (7): Anti-fungal activity of the purified chitinase against P. grisea using different concentrations on media. The arrows indicate the hyphal extension inhibition zones.

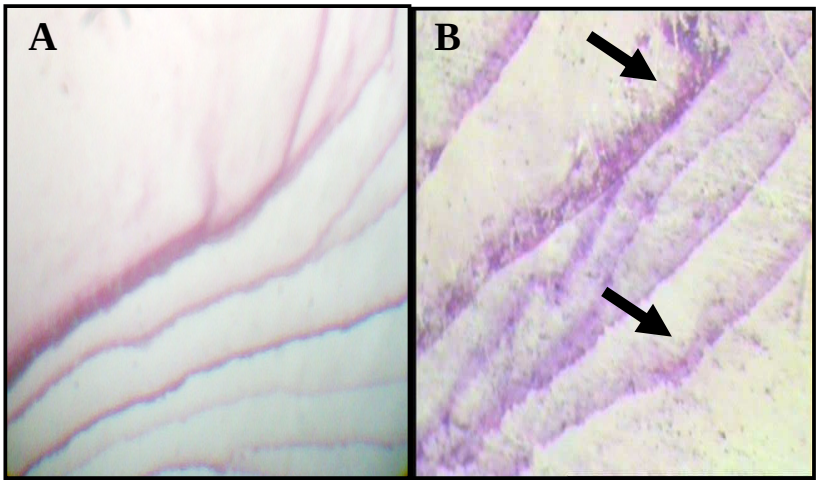

Fig.(8): Microscopic analysis of chitinolytic activity on fungal mycelium (B) compared to healthy fungal (A). The arrows point to mycelium deformation of the fungus and inhibition of mycelial branching.

DISCUSSION

Agricultural crops undergo to an enormous collection of fungal diseases which cause severe yield losses. As a consequence of fungal plant diseases, the yield losses in crop plants may reach about 25% in western countries and almost 50% in developing countries. The interaction between a pathogen and a plant initiates a complex network of defense mechanisms, among which is a dramatic increase in constitutive overex-pression pathogenesis-related (PR) proteins. of PR proteins (Schickler and Chet, 1997). Among these anti-fungal proteins, chitinase belonging to the PR group of proteins, appears to be a potential candidate for disease resistance. Furthermore, plants have evolved a number of defense responses are elicited during their life cycle in response to developmental signals and pathogen attack (Vigers *et al*., 1991; Swegle *et al*., 1992).

In plants and animals, chitinases mainly play a role in the defense of the organism against pathogen attack, but they can also be involved in various growth and developmental processes. Consequently, the main goal of this work was to isolate and clone the *chitinase* gene in an expression system to investigation the chitinase biological activity against *P. grisea* growth and development.

Many authors reported the cloning and purification of chitinase from different plants. Roberts and Selitrennikoff (1988) studied plant and bacterial chitinases for antifungal activity. According to them, chitinases isolated from the grains of wheat, barley and maize functioned as endochitinases and inhibited hyphal elongation of test fungi. In addition, chitinase, purified from thorn apple, tobacco and wheat, inhibited growth of some saprophytic fungi (Broekaert *et al*., 1988). Also, Radjacommare *et al*. (2004) described the purification of 57 kDa chitinase from *P. grisea* challenged finger millet plants pretreated with *Pseudomonas fluorescens* strain. In addition, a chitinase gene from barley was cloned and overexpressed in *E. coli*. Chitinase (35 kDa) was isolated and purified. Purified chitinase exerted broadspectrum antifungal activity against many fungi. Due to the potential of broad-

spectrum antifungal activity, chitinase gene can be used to enhance fungal-resistance in crop plants such as rice, tobacco, tea and clover (Kirubakaran and Sakthivel, 2007).

In the case of rice (*Oryza sativa*), class II chitinase CHT11 was identified in rice (*Oryza sativa*), cloned and overexpressed in *E. coli* BL21 cells as a glutathione-S-transferase (GST) fusion protein (Xayphakatsa *et al*., 2008). In addition, acidic and basic class III chitinases from rice (*Oryza sativa* L.) were expressed from the corresponding cDNAs in *Pichia pastoris* and homogenously purified (Park *et al*., 2002). In the present study, the *chitinase* gene had been identified and isolated from Egyptian rice cultivar. The size of *chitinase* gene was found to be approximately 1023 bp, and encoded a protein composed of 340 amino acids. SDS-PAGE revealed that the level of chitinase expression had increased from 16 to 48 hr post infection. The highest expression of chitinase was observed after 24 hr. The present results are in agreement with those obtained by Kim *et al.* (2003). They reported that PR chitinase induced by *P. grisea* had been repeatedly observed 24 hr after inoculation. In fact, *P. grisea* conidia germinate within 30 minutes of attachment upon receiving host inductive signals, appressoria form between 4-8 hr and penetration pegs form approximately 24 hr after inoculation (Talbot, 2003). On the other hand, the timing of chitinase expression during the early phase of invasive growth of *P. grisea* is largely unknown (Zhong-hua *et al.*, 2007). A study of the time required for initiating an active defense response to the pathogen *P. grisea* will accelerate the development of new approaches to produce durably blast resistant rice cultivars.

The purified protein reported in this study (~35 kDa) showed *in vitro* antifungal activity against *P. grisea.* The chitinase activity assay and SLM examination disclosed chitinase-induced lysis and fragmentation of the mycelium and hyphal distortion in the fungus. As a result, chitinase affects the *P. grisea* growth and development.

To discuss the previous results, many reports suggested that chitinases have a role in plant defense by degrading the fungal cell walls. They catalyse the hydrolytic cleavage of the β-1,4-glycoside bond present in the biopolymer of N-acetyl glucosamine (chitin) found in fungal cell walls (Keefe *et al*., 1990; Cohen-Kupiec and Chet, 1998, Xayphakatsa *et al*., 2008). Microscopic observations of these test fungi revealed deformed hyphae with reduced hyphal branching, fragmentation and lysis of cells (Kirubakaran and Sakthivel 2007). As well, chitinases hydrolyse chitin which is a major component of fungal cell walls. Chitin and glucan oligomers, released during degradation of fungal cell wall, elicit various defence mechanisms in the plants (Frindlender *et al*. 1993). Velazhahan *et al*. (2000) reported a rapid increase in chitinase activity in rice cells due to elicitor treatment which possessed anti-fungal property and suggested that chitinase plays a role in disease resistance of rice. In the present study, it was observed that the chitinase purified from rice inhibits the growth of *P. grisea*. The gene coding for such chitinase could be highly useful for developing disease resistant.

Because many chitinases are located in extracellular and/or intercellular space, their possible role in plant defense might be to act as signaling molecules, releasing elicitors from invading fungal hyphae and acting as a first line of defense (Mauch and Staehelin, 1989). In higher plants, PR-3 chitinases are considered to play an important role in protection of plants from the invasion of pathogens, because PR-3 chitinases are known to inhibit fungal growth both *in vitro* (Sela-Buurlage *et al*., 1993) and *in vivo* (Anand *et al*., 2004). Generally, gene expression and

enzymatic activity of chitinases are activated by pathogenic invasion (Ferreira *et al*., 2007).

Chitinase exhibited an antifungal activity against phytopathogenic fungi and therefore, can be used to develop fungal resistant crop plants such as rice and other plants. Results presented in this study will be useful to design appropriate strategies for transgenic resistant plants, and may be useful to manufacture bio-antifungal agents.

REFERENCES

Anand, A.; Lei, Z.; Sumner, L.W.; Mysore, K.S.; Arakane, Y. and Bockus, W.W. (2004). Apoplastic extracts from a transgenic wheat line exhibiting lesion-mimic phenotype have multiple pathogenesis-related proteins that are antifungal. Mol. Plant Microbe Inter., 17:1306–1317.

Bowles, D. (1990). Defense-related proteins in higher plants, Annu. Rev.Biochem., 59: 873–907.

Broekaert, W.F.; Van Parijis, J.; Allen, A.K. and Peumans, W.J. (1988). Comparison of some molecular enzymatic and anti-fungal properties of chitinases from thorn apple, tobacco and wheat. Physiol. Mol. Plant Pathol., 33:319–331.

Broglie, K.; Chet, I.; Holliday, R.; Cresmann, M.; Biddle, P.; Knowlton, S.; Mauvais, C.J. and Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science, 254:1194–1197.

Cao, H.; Li, X. and Dong, X. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. USA, 95:6532–6536.

Coca, M.; Penas, G.; Campo, J.G.S.; Bortolotti, C.; Segundo, J.M.B. (2006). Enhanced resistance to the rice blast fungus *Magnaporthe grisea* conferred by expression of a *cecropin A* gene in transgenic rice. Planta, 223: 392–406.

Cohen-Kupiec, R. and Chet, I. (1998). The molecular biology of chitin digestion. Curr. Opin. Biotechnol., 9270–277.

Datta, S.; Muthukrisnan, S. and Datta, S.K. (1999). Expression and function of PR proteins in transgenic plants. In: Datta S.K. and Muthudrishan, S. (eds) Pathogenesis-related proteins in plants. CRC Press, New York, pp 261–277.

Ferreira, R.B.; Monteiro, S.; Freitas, R.; Santos, C.; Chen, Z.h.J. and Batista, L.M. (2007). The role of plant defence proteins in fungal pathogenesis. Mol. Plant Pathol., 8:677–700.

Frindlender, M.; Inbar, J. and Chet, I. (1993). Biological control of soil borne plant pathogens by a β-1,3 glucanase producing *Pseudomonas cepacia*. Soil Biology and Biochemistry, 25:1211–1221.

Johnson, L.F. and Curl, E.A. (1972). Methods for research on the ecology of soil borne plant pathogens, Burgess Publishing Company, Minnesota, p. 241.

Keefe, D.; Hinz, U. and Meins, F. (1990). The effect of ethylene on the cell-type specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. Planta, 182:43–51.

Kim, S.T.; Cho, K.S.; Yu, S.; Kim, S.G.; Hong, J.C.; Han, C.; Bae, D.W.; Nam, M.H. and Kang, K. Y. (2003). Proteomic analysis of differentially expressed proteins induced by rice blast fungus and elicitor in suspension-cultured rice cells. Proxemics, 3:2368–2378.

Kirubakaran, S. I. and Sakthivel, N. (2007). Cloning and overexpression of antifungal chitinase gene in *Escherichia coli.* Protein Expression and Purification, 52:159–166.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680–685.

Lorito, M.; Woo, S.L.; Garcı´a-Fernandez, I.; Colucci, G.; Harman, G.E.; Pintor-Toro, J.A.; Filippone, E.; Muccifora, S.; Lawrence, C.B.; Zoina, A.; Tuzun, S. and Scala, F. (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA, 95:7860–7865.

Mauch, F. and Staehelin, A. (1989). Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant cell, 1:447–57.

Ou, S.H. (1985). Rice blast. Rice Disease. 2nd ed, The Cambrian News Ltd., U.K, pp 109-201.

Park, S.; Kim, D.; Truong, N.H. and Itoh, Y. (2002). Heterologous expression and characterization of class III chitinases from rice (*Oryza sativa* L.). Enzyme and Microbial Technology, 30:697–702.

Pappu, H.; Pappu, S.; Jain, R.; Bertrand, P.; Culbreath, A.; Mcpherson, R. and Csinos, A. (1998). Sequence characteristics of natural populations of tomato spotted wilt tospovirus infecting flue-cured tobacco in Georgia. Virus Genes 17: 169-177.

Radjacommare, R.; Ramanathan, A.; Kandan, A.; Sible, G.V.; Harish, S. and Samiyappan, R. (2004). Purification and anti-fungal activity of chitinase against *Pyricularia grisea* in finger millet. World Journal of Microbiology and Biotechnology, 20:251–256.

Roberts, W.K. and Selitrennikoff, C.P. (1988). Plant and bacterial chitinases diver in antifungal activity. J. Gen. Microbiol., 134:169–176.

Roy-Barman, S. and Chattoo, B. B. (2005). Rice blast fungus sequenced. Current Science, 89(6):930-931.

Schickler, H. and Chet, I. (1997). Heterologous *chitinase* gene expression to improve plant defense against phytopathogenic fungi. J. Ind. Microbiol. Biotechnol., 19:196–201.

Sela-Buurlage, M.B.; Ponstein, A.S.; Bres-Vloemans, S.A.; Melchers, L.S.; van den Elzen, P.J.M. and Cornelissen, B.J.C. (1993). Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol., 101:857–63.

Swegle, M.; Kramer, K.J. and Muthukrishnan, S. (1992). Properties of barley seed chitinase and release of embryo-associated isoforms during early stages of inhibition. Plant Physiology, 99:1009–1014.

Talbot, N. J. (2003). On the trail of a cereal killer: investigating the biology of *Magnaporthe grisea*. Annu. Rev. Microbiol., 57:177–202.

Valent, B. and Chumley, F. G. (1991). Molecular genetic analysis of the rice blast fungus *Magnaporthe grisea*. Annu. Rev. Phytopathol., 29:443–467.

Velazhahan, R.; Samiyappan, R and Vidhyasekaran, P. (2000). Purification of an Elicitor-inducible anti-fungal chitinase from suspensioncultured rice cells. Phytoparasitica, 28:131–139.

Vigers, A.J.; Roberts, W.K. and Selitrennikoff, C.P. (1991). A new family of plant anti-fungal proteins. Molecular Plant Microbe Interaction, 4:315–323.

Xayphakatsaa, K.; Tsukiyamaa, T.; Inouyeb, K.; Okumotoa, Y.; Nakazakia, T. and Tanisaka, T. (2008). Gene cloning, expression, purification and characterization of rice (*Oryza sativa* L.) *class II chitinase CHT11*. Enzyme and Microbial Technology, 43:19–24.

Zeigler, R. S.; Leong, S. A. and Teeng, P. S. (1994). Rice Blast Disease. CAB International, Wallingford.

Zhong-Hua,W.; Yu-Lin, J.; Hui, L.; Intern, A.; Valent, B. and Rutger, J. N. (2007). Host active defense responses occur within 24 hours after pathogen inoculation in the rice blast system. Rice Science, 14(4):302-310.

الملخص العربي

التعبير الفائق لجين كيتينز الأرز و تقييم قدرة الإنزيم كمضاد حيوي للفطريات

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تم عزل و تنقية سبع عزلات لفطر *P. grisea* من أوراق الأرز المصابة, وعلى فترات زمنيه 16,20,24,48 ساعة مع استخدام تكنيك SDS-PAGE لاستخلاص البروتينات الكلية من الأوراق المصابة والسليمة لنباتات الأرز. واظهر التكنيك SDS-PAGE أن حزمة بروتين الكيتينز عند 35 كيلو دالتون زادت كثافتها بعد مرور 24 ساعة من الإصابة الفطرية. وتم استخدام تكنيك RT-PCR لعمل تضاعف للتتابع المقروء لجين الكيتينز وأمكن الحصول على شظية طولها 1023 زوج من القواعد , وتم عمل كلونه لهذه الشظية في نظام تعبيري ببكتريا القولون *E. Coli* وبواسطة عمود كروماتوجرافي Ni-NTA أمكن استخلاص الناتج البروتيني (الكيتينز). وأظهرت النتائج قدرة ونشاط الكيتينز المستخلص كمضاد لنمو الفطر *P. grisea*.