Isolation of chitinase gene induced during infection of *Vicia faba* by *Botrytis fabae*

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ABSTRACT

The moderately resistant (Giza 716) and the susceptible (Giza 429) faba bean cultivars were used to identify some pathogenesis related proteins (PRs) associated with infection by chocolate spot disease. One isolate of Botrytis fabae purified from a plant sample taken from Nubaria location (Behera governorate, Egypt) was used in the artificial infection experiment. Qualitative and quantitative analyses were carried out on all protein banding patterns of the healthy and the infected faba bean leaves harvested at 8, 24 and 48 hr after inoculation. Data revealed that a 26 kDa protein band was more intensive 8, 24 and 48 hr after inoculation in cultivar Giza 716,. In addition, a 29 kDa protein band appeared after 24 and 48 hr. Furthermore, in cultivar Giza 429, 54 kDa protein bands appeared after 8, 24 and 48 hr post inoculation and 28 and 20 kDa appeared after 24 hr post inoculation.Reverse-Transcription (RT-PCR) showed that chitinase gene is expressed at very early stages in infected faba bean leaves. DNA fragment at molecular weight 900 bp appeared at 8, 24 and 48 hr after inoculation and disappeared in the healthy plants. The amplified products were cloned into pGEM-T Easy vector. Four clones named (PNAM1, PNAM2, PNAM3 and PNAM4) were selected for validation. The recombinant plasmids PNAM1, PNAM2 were verified for the presence of the Chitinase gene coding sequences by using both specific and universal primers in PCR. BNAM1-Chit-EG gene sequence showed 58.15% similarity when aligned with other Chitinase genes published in the gene bank.

***Key words**: Vicia faba, Botrytis fabae, pathogensis- related protein, clonin , chitinase gene, sequencing.*

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most important food legumes in Egypt. This crop is attacked by a number of plant pathogens and parasites. The most important fungal disease is chocolate spot. The causal organisms of chocolate spot are *Botrytis fabae* and *Botrytis cinerea.* Although both species are able to cause the disease in the field, *Botrytis fabae* is more aggressive than *Botrytis cinerea*. Chocolate spot is the most destructive disease and causes considerable losses in the yield of faba bean in the northern region of the Nile Delta of Egypt, where low temperature and high relative humidity favoring its spread are prevailing (Mohamed, 1982).

Inoculation of plants with compatible and incompatible pathogens triggers a variety of plant defense responses, including the

activation of genes encoding pathogensis-related proteins (Van Loon, 1997). Many of these proteins have been shown to exhibit antifungal activity *in vitro* (Ponstein *et al.*, 1994), for example the PR2-class (*B*-1, 3 glucanase) and PR3 class (chitinase) proteins hydrolyzing the important fungal cell wall components (Mauch *et al.*, 1988). This suggested that PR proteins may paly an important role in pathogen defense. Some of the tobacco PR-proteins were identified as chitinase (Legrand *et al.*, 1987) and *B* 1,3 glucanase (Kauffmann *et al.*, 1987) with potential antifungal activity, it has often been suggested that the collective set of PR-proteins may be effective in inhibiting pathogen growth, multiplication and spread (Kombrink and Somssich, 1997). Plant disease resistance genes (R genes) encode proteins that detect pathogens. The tools of biochemical and molecular studies have been applied to investigate the mechanisms involved in disease resistance. There have been experimental successes with foreign proteins in plants to develop disease resistant crops.Van Loon (1985) reported that the biochemical changes occurring in the stressed plant include the production of pathogenesis – related proteins (PR), among these are chitinases. These enzymes are capable of catalyzing chitin containing fungal cell wall and is therefore may play a major role in the plant response (Schickler and Chet, 2004). Mauch *et al* .(1988) proved that a basic chitinase in combination with a basic *B*-1, glucanase, both isolated from bean plants, have a strong antifungal effect *in vitro*. Margis-Pinherio *et al*. (1991) stated that two acidic chitinases were formerly called bean PR3 and PR4 proteins or chitinases. Danhash *et al*. (1993) studied the molecular character-rization of four chitinase cDNAs obtained from *Cladosporium fulvum* infected tomato. They reported that the southern blot analysis of tomato chitinase confirmed that the acidic extracellular 26 and 27 kDa chitinase are each encoded by a single gene in tomato. Mahe *et al*. (1993) observed that the first induction of chitinase mRNA occurred 8-10 hr, before hypersensitive response symptoms were visible in *Colletotrichum lindemuthianum* bean interaction. Furthermore, Margis-Pinherio *et al*. (1993) reported that chitinase mRNA became detectable after a few hours post inoculation. Enan *et al.* (1994) found two polypeptides (26 kDa protein band and 16 kDa protein band) in protein banding pattern of infected faba bean cultivar (Giza 402) during infection with *Botrytis fabae* using SDS-PAGE. These two polypeptides may be pathogenesis – related proteins (PRs) induced in response to fungal infection (10-12 hr. after inoculation) before the onset of lesion formation in bean plants. Lingrang Kong *et al*. (2005) constructed a cDNA library using mRNA isolated from wheat plants harvested at 2,6,12,24,36,72 and 96 hr. after inoculation with conidiospore suspension of *Fusarium graminearum*. They added that the cDNA clone encodes an acidic isoform of class 1 chitinase containing 960 bp coding region.

The present work was planned to identify PR proteins induced by infection with *Botrytis fabae* in faba bean and also to clone and sequence the genetic regions responsible for these types of proteins.

MATERIALS AND METHODS

Plant cultivars

Two cultivars of faba bean were used in this study as a host plant for the artificial infection with *Botrytis fabae* isolate. Both cultivars were obtained from Field Crop Research Institute, Agricultural Research Center, Giza, Egypt. The response of these cultivars to chocolate spot disease are different, i.e., Giza 429 cultivar is highly susceptible and Giza 716 cultivar is moderately resistance.

Fungal isolates

One aggressive *Botrytis fabae* isolate was used in the present study for the artificial infection, isolated from Nubaria location, Behera governorate.

Artificial infection

Faba bean plants **(**45 days old) were artificially infected with the spore suspension (25 x 10^4 spores/ml) of the isolate under study, using a fine mist hand sprayer. Inoculated plants were then covered with polyethylene sheets supported with metal frames to maintain a high relative humidity. Samples were taken after 8, 24 and 48 hr post inoculations, respectively.

SDS-Polyacrylamide gel electrophopresis

Two grams from each plant leaves were taken and ground in liquid nitrogen to a fine powder, 50 mg of the ground sample were transferred to an eppendorf tube and 0.7 ml of extraction buffer (0.6 ml 1 M Tris HCL, pH 6.8, 5 ml 50 % glycerol , 2 ml 10 % SDS , 0.5ml β -mercaptoethanol ,and 0.9 ml H_2O) was added . The extract was centrifuged at 14000 rpm for 15 min under cooling. Supernatants containing soluble protein fractions were transferred to clean tubes and stored at - 20 °C. Protein content was estimated according to the methods of Bradford (1976) by using Bovine Serum Albumin (BSA) as a standard. Protein content was adjusted to 2 mg / ml per sample. SDS was added to the sample at the rate of 4 mg SDS / 1 mg protein, then 50 µl, β mercaptoethanol were added. The mixture was boiled at 100 °C in a water bath for 3-5 min and 20 µl of this crude protein solution were resolved on 12 % SDS – PAGE using molecular weight protein marker as a standard. Electrophoresis was carried out at 2 milliampere per sample till the samples reach one inch from the bottom of the gel. Gels were removed from the apparatus and placed in plastic tanks containing 50 % ethanol and 10 % acetic acid-freshly prepared, then gels were stained by the silver staining method for protein described by Sammons *et al.* (1981). Gels were scanned using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631). The different molecular weights of each band were determined.

Amplification of chitinase gene by RT-PCR

Total RNA was isolated from healthy and infected plants after different time intervals, i.e., 8, 24 and 48 hr post inoculation using the method described by Enan *et al.* (1994). RNA was purified using tri- reagent RNA kit (Sigma). cDNA synthesis was carried out in a reaction mixture (40 µl final volume) containing 10 µl of freshly prepared RNA for template: 10 pmol of oligo(dt) antisense primer : 20 U of M-MuLV reverse transcriptase (Promega) : 100 mM of each dNTP; 1 mM DTT; 50 mM Tris – HCL. pH 8.3; 75 mM KCL and 6 mM $MgCL_2$. The reaction mixture was incubated at 37°C for 1 hr and stored at -20°C until use. Chitinase specific products were amplified by PCR, (using *T-GRADIENT* thermal cycler from Biometra) in 25 µl volume containing 2.5 µl of cDNA; 25 pmol of each primer forward primer (Chit 1) 5`-ATT ATT GTT CTT TTA GTC CT-3` and reverse (Chit 2) 5`-CTT TGT TCT TAT TCC ATT GA-3` ; 10 mM of each dNTPs; 1 U of Taq DNA polymerase; 10 mM Tris-Hcl, pH 9.0; 50 mM Kcl; 1 mM $MgCl_2$. Denaturation at 94 ºC for 3 min was followed by 35 cycles of 1 min at 94ºC; 2 min at 45ºC and 1 min at 72 ºC with a final extension step at 72º C for 7 min , for a total. Conditions for the amplification of hemi-nested products were the same, except that 25 pmol of specific primers (Chit 1) and (Chit3) 5`-GGCGGCACGGGTA GGGGTGACATTG-3` and 1 µl of 1000 folds diluted first PCR reaction mixture for template were used. Three oligonucleotide primers were

synthesized according to the published sequence clone PHs2 Gene Bank Accession No. L22032.

Cloning of chitinase into pGEM-T-Easy vector

The generated DNA fragments of chitinase genes obtained after PCR amplification were purified using QiA quick gel extraction kit (Qiagen) according to manufacturer's instructions; the products were ligated into pGEM- T Easy vector (system 1) from Promega (Madison,WI, USA) , and transformed into competent JM 109 *E. coli* cells (Promega) with subsequent ampicillin selection following manufacturer's instructions.

The promega pGEM- T Easy cloning kit offered a rapid and efficient cloning method for PCR products based on the use of the T overhang in the linearized vector and A tail on the PCR products as an alternative to the DNA overhangs. 2 µl of ligation reactions were add and mixed gently by tapping and incubated on ice for 30 min. Cells were heat shocked for 45 sec. at 42ºC in a water bath incubator to increase the transformation efficiency. The tubes were then placed on ice for 1 min to cool down, 900 µl of LB medium were added to each tube and shaked gently at 37ºC for 2 h for cell recovery. 100 µl transformation mix were plated onto Luria-Bertani (LB) plates containing ampicillin 50 mg/ml ,100 mM IPTG and 50 ug/ml X-gal and incubated at 37ºC overnight for selecting the transformed cells. The sample cultures were grown overnight at 37ºC. Plasmid DNA was prepared from each culture by using the QiA prep spin, Mini prep kit (Qiagene), following manufacturer's instructions.

DNA sequencing

The recombinant plasmid (PNAM1) containing chitinase fragment was recultured separately in 5 ml LB medium with ampicillin at 100 ug/ml. Plasmid was extracted using QiA prep® Miniprep kit (Qiagen). The DNA insert was verified by (1) PCR using M13F/ M13R and Chit F/ Chit R primers to validate cloning. (2) Sequencing of one strand on an Applied Biosystems 310 genetic analyzer. (Applied Biosystems, ABI) using sequencing redy reaction mix according to manufacturer's instruction (Applied Biosystem) at Gene Analysis Unit, VACSERA.

RESULTS AND DISCUSSION

In order to find biochemical differences between the healthy and the infected plants at different intervals, total protein extracts were isolated and electrophoresed on one dimension (SDS-PAGE).

Differences in the intensity of the same protein bands between the infected and healthy plants obtained at molecular weight of 26 kDa after 8, 24 and 48 hr post inoculation, which was very weak in the healthy plants, while a new protein band at molecular weight of 29 kDa presented in the infected plants after 24 and 48 hr. post inoculation, as shown in (Fig. 1). This band agrees with that obtained by Enan *et al*. (1994). Protein pattern of faba bean leaves (cultivar Giza 429) at different periods post inoculation with *Botrytis fabae* Nubaria isolate (Fig. 2) revealed that a new protein band at a molecular weight of 54 kDa appeared after 8, 24 and 48 hr post inoculation and 28 and 20 kDa appeared after 24 hr post inoculation.

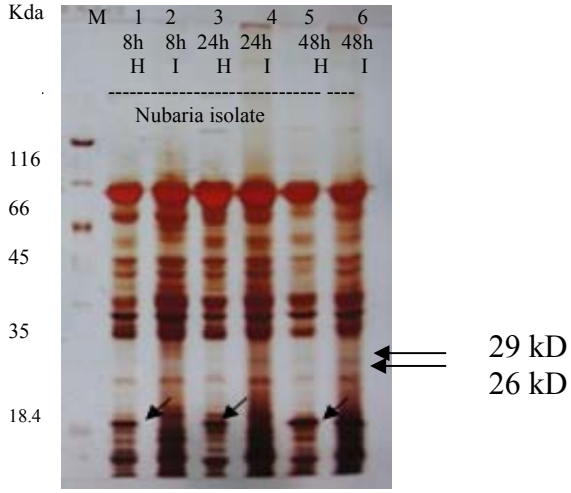

Fig (1): SDS – PAGE showing the protein patterns analyzed after 8, 24 and 48 hr of healthy (H) and infected (I) faba bean cultivar (Giza 716) with Botrytis fabae Nubaria isolate (lanes 1-6). M mid molecular weight protein marker.

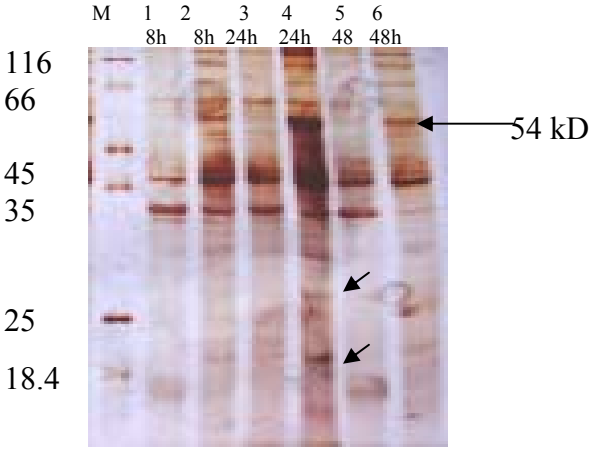

Fig (2): SDS – PAGE showing the protein patterns analyzed after 8, 24 and 48 hr of healthy (H) and infected (I) faba bean cultivar (Giza 429) with Botrytis fabae Nubaria isolate (lanes 1-6). M mid molecular weight protein marker.

Upon interaction with the pathogen, plants initiate a complex network of defense mechanisms, among which is the dramatic increase in chitinase activity. Isolation and characterization of disease-related genes may need to be complemented by biochemical studies to understand fully their functions (Kotchoni and Shonukan, 2002). Electrophoretic patterns of soluble proteins have been used as a powerful tool for the study of genetic variability of infected and healthy plants. In addition, various novel proteins are collectively referred to as pathogenesis-related proteins. These PRs defined as proteins coded for by the host plant, but induced specifically in pathological or related situations.

Chitinase enzyme is an important component in eukaryotic signal transduction pathway. A gene from faba bean plants (cultivar Giza 716) was characterized after infection with *Botrytis fabae* (Nubaria isolate) during the present investigation. The quality of RNA isolated during this study depended on the source of tissue being used as a starting material. The protocol described under the materials and methods (Enan *et al*. 1994) was used successfully to isolate a high yield of total RNAs from infected and healthy leave tissues. The RNA was reverse transcribed by the M-MLV reverse transcriptase using oligo (dt) as

minus-sense primer and the resulting complementary DNA (cDNA) was amplified by PCR after adding Chit1 and Chit2 primers to amplify 900 bp fragment in the infected plants, where this band disappeared in the healthy plants (Fig. 4). Meanwhile, Chit1 and Chit3 primers were used to amplify 340 bp fragment (Fig. 5). These results were in agreement with those obtained by (Mahe *et al*.(1993); Margis-Pinheiro *et al.* (1993) and Lingrang Kong *et al.* (2005).

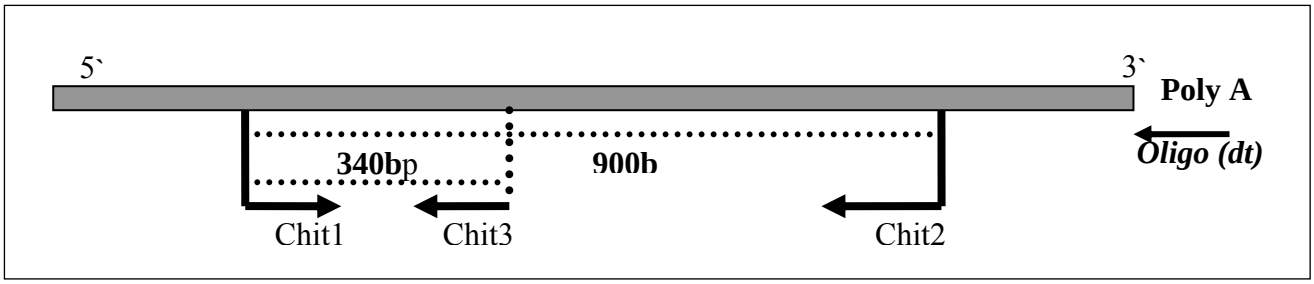

Fig. (3): Cistron map of the clone HPS2. Primers used for PCR amplification of partial sequences from chitinase gene are indicated by arrows. Chit1 with Chit2 gave a PCR fragment size 900 bp and Chit1 with Chit3 gave a PCR fragment size of 340 bp. Oligo (dt) (Roche) primer was used in reverse transcription reaction as minus primer.

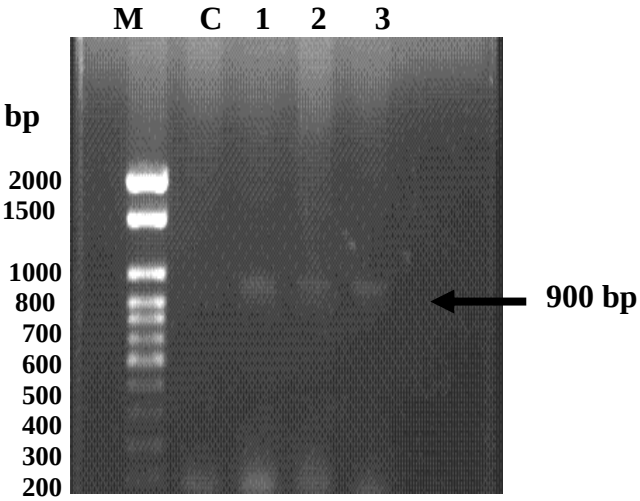

Fig. (4): Agarose gel electrophoresis showing the RT-PCR products of the chitinase genes region of faba bean using Hi-expand –Fidelity PCR system. 200 ng of total RNA extracted from infected faba bean tissue reverse transcribed into cDNA using oligo (dt) (Roche) minus primer. Lanes (1 ,2 and 3) : RT-PCR products of correct size (900 bp) at 8, 24 and 48 hr. after inoculation, respectively amplified using Chit1 and Chit2 primers. lane C: healthy faba bean tissue . Lane M: Molecular weight marker (100 bp)

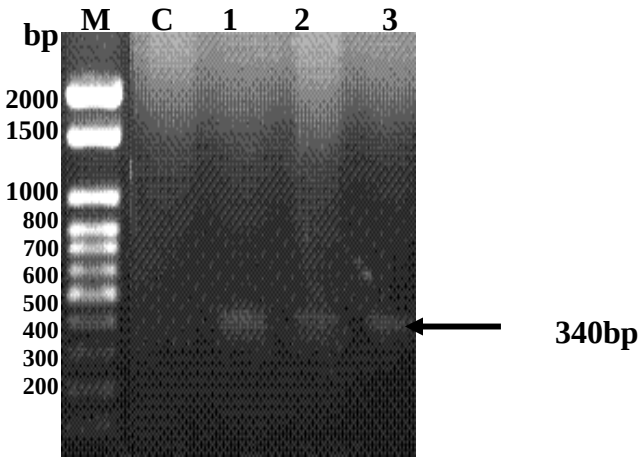

Fig. (5): Agarose gel electrophoresis showing the RT-PCR products of the chitinase gene of faba bean using Hi-expand–Fidelity PCR system.200 ng of total RNA extracted from infected faba bean tissue reverse transcribed into cDNA using oligo (dt) minus primer. Lanes (1,2 and 3): RT-PCR products of correct size (340 bp) at 8, 24 and 48 hr. after inoculation respectively amplified using Chit1 and Chit3 primers . Lane C: healthy faba bean tissue . Lane M: Molecular weight marker (100 bp).

Four white colonies *named (PNAM1, PNAM2, PNAM3 and PNAM4)* lanes1-4 resistant to ampicillin containing recombinant plasmids were selected for testing the presence of the chitinase gene by PCR. Plasmids were amplified using both Chit1 and Chit2 and M13F /M13R primer pairs as shown in (Fig. (6). The recombinant clones using the primer pair M13 forward and M13 reverse showed amplified product of size 1700 bp, where it amplified 900 bp when using Chit1 and Chit2 primers.

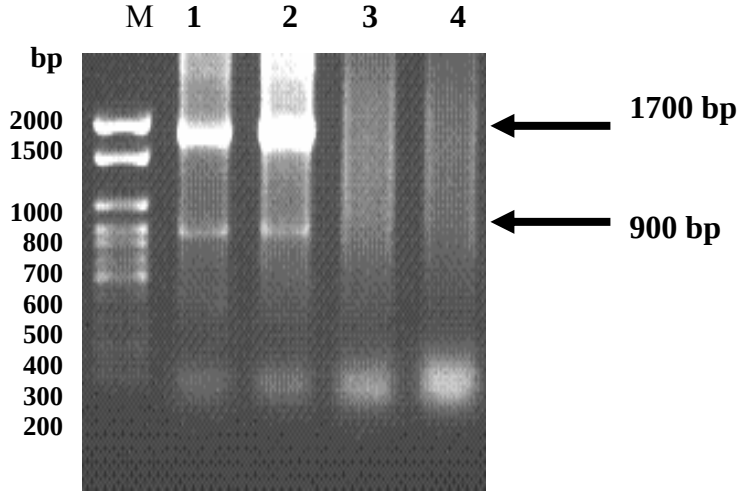

Fig. (6): Agarose gel electrophoresis showing the PCR products after cloning into PGEM-T-Easy vector using Hi-expand –Fidelity PCR system. PCR wes performed on recombinant chit clone to verify the presence of chitinase gene insert in PGEM-T-Easy vector. Lanes (1 and 2) showing the expected size (1.7 kb) of the amplified PCR product using M13 universal primers, and (900 bp) as the expected size of the amplified PCR with specific primers (Chit1 and Chit2).

Sequencing and alignment analysis of *BNAM1* clone

BNAM1 clone was sequenced using forward primer used in RT-PCR technique. Using DNAMAN V 5.2.9 package, Madison, Wisconsin, USA, the sequence obtained from *BNAM1* clone was aligned to the published chitinase sequences in gene bank.

Comparison of partial nucleotide sequence of *BNAM1-Chit-EG* showed 58.15% sequence homology with the other published sequences of Chitinase genes under the accession numbers (L22032 and DQ078281 of *Ulmus americana* Chitinases); (L16798, of *Zea mays* class I acidic Chitinase); (U97522, of *Vitis vinifera* class IV *endochitinase);* (D45183, *Chenopodium amaranticolor* Chitinase); (AF112965, *Triticum aestivum* (bread wheat) beta-1,3-glucanase precursor) as shown in Fig. (7).

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L22032          TATCAAGCTCC----GCCTTTAATGACATGCTTAAACAT---CGTAACGACCGGTGGTTT
DQ078281        TATCAAGCTCC----GCCTTTAATGACATGCTTAAACAT---CGTAACGACCGGTGGTTT
F.bean Chit-Eg  NNNTNNNTNNCNGGCTTCNTCAGAAGNGNNGNAACTGGA---TGTNCC-TTCCGGGNGAG
Chit-Chenopod   AGTTTGGTGACAGACGCGTTCTTTAATGGGATTATTAAC---CAAGCAGGCTCTAGCTGT
Chit-Vitis      GATATTGTGACACAGGCATTTTTCGATGGGATAATTAAT---CAAGCTGCTTCGAGTTGT
Chit-Maize      TATCACAGAAGAT--CTCTTCGAGAGGATGCTGAAGCAC---CGCAACGAACCTGACTGC
AF112965        GGCACGGGCATCAGCCTCCTCATGGACGTCGGCAACGGCGCGCTAACCAGCCTCGCAAAC
                                     *           *

L22032          CCTGCCAAGGGGTTTTACACCTATGATGCT-TTTATTTCGGGGCTGTCCAAGGCGTTTCC
DQ078281        CCTGCCAAGGGGTTTTACACCTATGATGCT-TTTATTTCGGGGCTGTCCAAGGCGTTTCC
F.bean Chit-Eg  ACTGTCGGGGAGTAAAATTGCCGTGTCGCCGTTGGTGCCCTCGCGCCCGTCAGAATGCGC
Chit-Chenopod   GCTGGTAAGAGGTTCTACACCAGATCTGCT-TTCTT-----GAATGCTCTCGGAAACTAT
Chit-Vitis      GCTGGGAAGAATTTTTACACCCGTGCAGCG-TTTCT-----CAGTGCCTTGAATTCGTAT
Chit-Maize      AAAGCTCGGGGCTTCTACACGTACGACGCC-TTCAT-----CACGGCGGCGGACGCGTTC
AF112965        GACCCCTCCGCCGCGCCCGCCTGGGTCAAGGCCAACGTGCAGCCCTTCCCGGGCGTCTCC

L22032          CTGCAATTTGC-----GGACCACCGGGGATGATATCACCCGTAAAAGGGAGATTGCTGCT
DQ078281        CTGCAATTTGC-----GGACCACCGGGGATGATATCACCCGTAAAAGGGAGATTGCTGCT
F.bean Chit-Eg  CATATGGTTA------GGGGCACTGGGTGGGCCNGC-CTCTCACAGACCACTTCAGTTNC
Chit-Chenopod   CCTCAGTTCG------GTAAAGGTGGATCCTCCGATGATACTAAGCGTGAAGTTGCCGCC
Chit-Vitis      TCTGGGTTCG------GCAACGATGGTTCTACCGATGCTAATAAGCGCGAGATTGCAGCT
Chit-Maize      CGGGGCTTCG------GCACCACGGGCAGCACGGAGGTCCAGAAGCGCGAGCTCGCCGCG
AF112965        TTCCGCTACATCGCCGTCGGCAACGAGGTCACGGACAGCGCCGGCCAGAAGACCATCCTC
                                        *                       *

L22032          TTCTTAGGCCAAACTTCCCATGAAACTACAGGGGGGTGGGCAAGTGCACCGAGGGGTCCA
DQ078281        TTCTTAGGCCAAACTTCCCATGAAACTACAGGGGGGTGGGCAAGTGCACCGAGGGGTCCA
F.bean Chit-Eg  GGCCCGATTCCAAAAGCCCAGCCGGCAATTGCC-------------------------
Chit-Chenopod   TTCTTTGCTCATGTCACCCATGAAACT-------------------------------
Chit-Vitis      TTCTTCGCTCATGTCACACACGAGACT-------------------------------
Chit-Maize      TTCTTGGGGCAGACGGGGCATGAGACCACGGGCGGCTGGCCGAACGCGCCCGACGGCGCC
AF112965        CCGGCCATAAAGAACATACAAACGGCGCTCGCGGCCGCCGGCCTCAGCGGCAGCATCAAG
                                  **     *

L22032          ATAACTCTTGGGGGATACTGCTACAATAGGGAGCCAAAAACCCTTCTTCTCGATTATTGT
DQ078281        ATAACTCTTGGGGGATACTGCTACAATAGGGAGCCAAAAACCCTTCTTCTCGATTATTGT
F.bean Chit-Eg  --------TTCTTGTTCTCGTCGAGCGTTGGACAGGGAGGGCNTCTGAC-CACTTATTTC
Chit-Chenopod   ---------GGAAGTTTTTGCTACATAGAGGAGATTTCGAAATC------TACCTATTGT
Chit-Vitis      ---------GGACACTTTTGTTATATTGAAGAAATCAATGGTGCCTCTCATAACTACTGT
Chit-Maize      TTCAC---CTGGGGCTACTGCTACAAGGAGGAGAACGGCGCCACCGCCGACTACTGCGAC
AF112965        GTGTCGACTTCGCTGCGGTTCGACGTGGTCAATAACACCTCCCCGCCCTCCAACGGCGTG
                                           *

L22032          TCTTTTAGTCC---TACT---TGGCCT--TGTGCTTCCGGAAAGAGATACTTTGGCCGTG
DQ078281        TCTTTTAGTCC---TACT---TGGCCT--TGTGCTTCCGGAAAGAGATACTTTGGCCGTG
F.bean Chit-Eg  TCGCCCAGCTC---CGACGGGCGGCTC--NCTNTTTCTGGCNT-AAACACCTTCATTACG
Chit-Chenopod   AACGCAAGCGC---AAC---ATGGCCG--TGCAATCCAAGCAAGCAATACTATGGCAGAG
Chit-Vitis      GATTCAAGCAA---TACCCAATATCCA--TGTGTCTCCGGTCAAAATTACTACGGCCGTG
Chit-Maize      ATGACGGGCGAGTACGCCCAGTGGCCG--TGCGTCGCCGGCAAGAAGTACTTCGGCCGCG
AF112965        TTCGCGGACACATCATTCATGGGGCCGATCCTGGACTTCCTGGCGAGCACCGGCGCACCG
                                        *                       **        *

L22032          GTCCCATTCAAC-TCTCCTGGAACTACAACTATGGACAGTGTGGAAGGCGC------ATA
DQ078281        GTCCCATTCAAC-TCTCCTGGAACTACAACTATGGACAGTGTGGAAGGCGC------ATA
F.bean Chit-Eg  TTCGCCTTCTGT-TTCCCATTCACTCTCCCCGTGGGGCCGTGCGCCTACTTN-----TTC
Chit-Chenopod   GGCCTCTTCAAC-TCACATGGAACTACAACTACGGAGCAGCCGGTAGAAGC------ATT
Chit-Vitis      GACCGCTTCAAC-TAACATGGAACTACAACTACGGCGCTGCTGGAAACAGC------ATT
Chit-Maize      GGCCCATCCAGC-TCTCCTACAACTACAACTACGGGCCGGCCGGGGAGGACGCGACCATC
AF112965        CTGCTGGTCAACGTGTACCCCTACTTCGCCTACAAGGGCGACCAGCAGAACATCAAGCTC
                         *    *         ***    *                          *

L22032          GGAGCAAACCTATTAAACAACCCTGATCTCGTAGCAACTGACCCTGTCATTTCCTTCAAA
DQ078281        GGAGCAAACCTATTAAACAACCCTGATCTCGTAGCAACTGACCCTGTCATTTCCTTCAAA
F.bean Chit-Eg  TAACTCAACATTCTCCNCNCTCTCGCGATTTGNTTTAACCAGGNTTTNANGCNCTAAATC
Chit-Chenopod   GGATTCGACGGTATTAATGCACCAGAAACAGTTGCTAACAACCCTGTTACTGCCTTTAGA
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Chit-Vitis      GGATTCAATGGCTTGAGCAACCCTGGAATTGTTGCAACTGACGTGGTTACTTCATTCAAG
Chit-Maize      GCCCAGGACCTGCTGAGCAACCCGGAGCTGGTGGCGTCGGACGCGTCCATCTCCTTCAAG
AF112965        GACTTCGCCACCTTCGTGCCAGGCAGCACCACCGTGACCGACAACGGGCTGACGTACAGC
                             *                                *           *  *

L22032          ACGGCCTTATGGTTCTGGATGA----------CCCCACAGTCA-------CCAAAGCCCT
DQ078281        ACGGCCTTATGGTTCTGGATGA----------CCCCACAGTCA-------CCAAAGCCCT
F.bean Chit-Eg  TCTATCTCTACTTCTTCCTTATCTNA----CTGCCTCANGTCATATCTGANCGAGGTCAT
Chit-Chenopod   ACAGCCTTCTGGTTTTGGATGAACAACGTCCACTCTATTATCAACTCCGGCCGAGGGTTC
Chit-Vitis      ACTGCATTATGGTTTTGGATGAATAATGTTCACTCTGTCCTAA------GCCAAGGTTTC
Chit-Maize      ACGGCCATCTGGTTCTCCATGA-------------CGGCGCAG-------CCCAAGCCGT
AF112965        AACCTGTTCGACGCCATGGTCGACTCCATCTACGCCGCACTGGAGAAAGCCGGCAAGCCC
                                  *

L22032          CGTGCCATGACGTCATCACCGGAAGATGGAGTCCT-TCCGGCACCGACCAGTCGGCCGGC
DQ078281        CGTGCCATGACGTCATCACCGGAAGATGGAGTCCT-TCCGGCACCGACCAGTCGGCCGGC
F.bean Chit-Eg  AG--CTATCCTTNTATCCTCTCATATAT--CTGCGATCTCATAGTGATCCTTANGTAANG
Chit-Chenopod   GGTGCCACCATTCGAGCTATCAATAGTA---TCGAATGTAATGGTGGTAATACAGGTGCT
Chit-Vitis      GGTGCCACAATTCAAGCCATCAATGGTGCCGTCGAATGTAATGGTGGAAACACAGCTGCC
Chit-Maize      CGTGCCACGACGTGCCAACCGAGCAGTGGACCCCC-TCGGCCGCCGACAAGGCCGCGGGG
AF112965        GACGTTAAGGTGGTCATATCCGAGAGCGGGTGGCCGTCGGCCGGTGGGGTCGGGGCGACG
                      *                                *       *       *

L22032          -CGAGTTGCGGGCTACGGCGTGATCACCAATATTATCAACG-GT--GGGATAGAATGCGG
DQ078281        -CGAGTTGCGGGCTACGGCGTGATCACCAATATTATCAACG-GT--GGGATAGAATGCGG
F.bean Chit-Eg  NAAAGCTAAAGTACCCTCTCCCCTCATCCCTTCANTCNACC-TCACTG-ATCTNCNATTA
Chit-Chenopod   GTCAATTCTCGGGTTCAACTCTATAGACAATATTGTAATCA-GTTTGGTGTTTCTCCTGG
Chit-Vitis      GTTAACGCCCGCGTTCAGTATTACAAGGACTACTGCAGTCA-GCTCGGGGTTTCACCTGG
Chit-Maize      -AGGCTTCCGGGCTACGGCGTCATCACCAACATCATCAACG-GC-----ATCGAGTGCGG
AF112965        GCGCAGAACGCGCGGGCTTACAACCAGGGATTGATCAACCACGTCCGCGGGGGCACGCCG
                                                        *

L22032          GAAAGGTCAGGTTCCCCAGGTGGTGGAACGGATTGGATTCTA-CAAAAGGTACTGTGATA
DQ078281        GAAAGGTCAGGTTCCCCAGGTGGTGGAACGGATTGGATTCTA-CAAAAGGTACTGTGATA
F.bean Chit-Eg  CAACACCAACTCTCCCTA-----CTATGCGCATCTTCTCCTCTCACGCGATTCCGTTCGA
Chit-Chenopod   GAACAATCTCAGTTGCTAAGTA--CATCTTTAATATAGTTTT-AAAGGTGTGTTGGTAGT
Chit-Vitis      TGACAACCTCACTTGCTGATAAGTCATACGTATACAAACTCC-AATTGAATTATACATAT
Chit-Maize      CAAAGGCTACAACGAGAAGGTGGCCAACCGGACCTTCTTCTA-CACCAGCTACTGCGACA
AF112965        AAGAAGCCCAGCTTGCTGG--AGACGTACATTTTCGCCATGTTCAACGAGAACCAGAAGA
                                                           *

L22032          TCCTTATAG---TTGGCTATGGGAACAACCTTGATTGCTATAACCAGAGGCCTTTTGGGA
DQ078281        TCCTTATAG---TTGGCTATGGGAACAACCTTGATTGCTATAACCAGAGGCCTTTTGGGA
F.bean Chit-Eg  CNAACATAA-----CGCCATGTTATGAAACATCNACCCTATGCTTTTCCTATTATAAGTC
Chit-Chenopod   ACTCTATGC-TACTAACCAACTTAAAAATTTTTTATTGTAATTTATGAAAAATAAAAGGA
Chit-Vitis      GAAGGGTATACGTAAACTATGGGAAGAATAAAGAGTTATGTGGTATGAAACCCTTAGTAG
Chit-Maize      TCCTTGGCA---TCAGCTACGGCGACAACTTGGACTGCTACAACCAGAGGCCTTCAACAG
AF112965        CAGGGGATC----CGAC--GGAGAACAACTTTGGGCTGTTCAATCCGGACAAGTCGCCGG
                               *         **          *

 L22032          ATGGACTCTTGTTGGACACCATGTAACGACT
 DQ078281        ATGGACTCTTGTTGGACACCATGTAA-----
 F.bean Chit-Eg  TCCTCACCNTTCTCCACTCCTCACCTTACAN
 Chit-Chenopod   T---ACCTGCTTAAAATACAATGAAATAAAA
 Chit-Vitis      T---ACTT--TTGCCATATAATGCTCTGCAT
 Chit-Maize      C-GCATCCTTGCTGGAACCGCTGCTCCGCTG
 AF112965        CCTACTCCGTTACTTTCTAAATGCAAAATTC
```

Fig. (7-A): CLUSTAL multiple sequence alignment of partial nucleotides encoding the Chitinase gene of the Egyptian faba bean (f. bean Chit-Eg) in comparison with the published Chitinase sequences. The overall sequences showed 58.15% sequence homology.

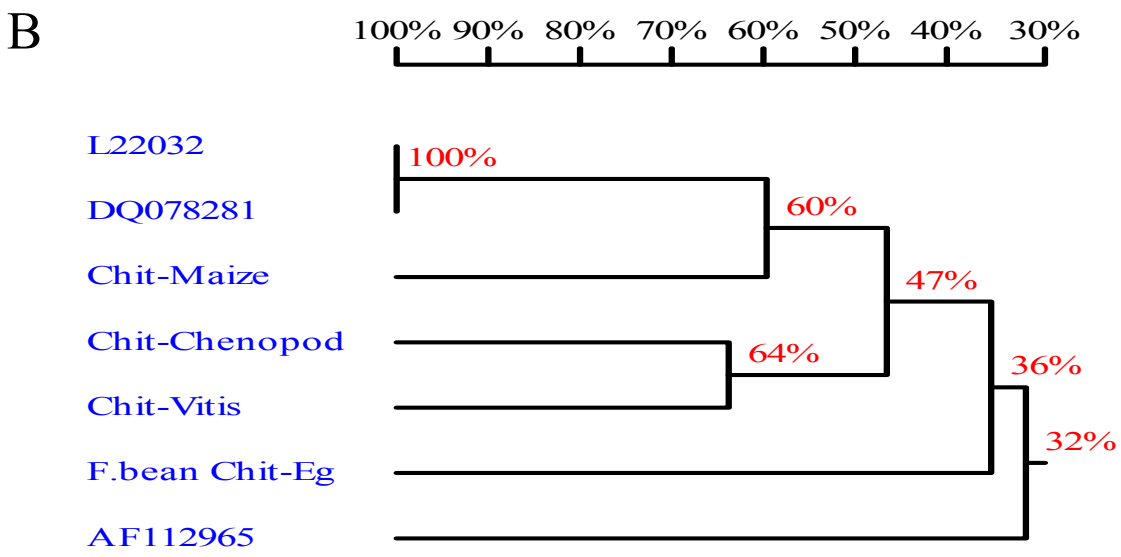

Fig. (7-B): CLUSTAL multiple sequence alignment of partial nucleotides encoding the Chitinase gene of the Egyptian faba bean Chitinase (F. bean Chit-Eg) in comparison with the published Chitinase sequences. The overall sequences showed 58.15% sequence homology (A). L22032 & DQ078281: Ulmus americana Chitinases, Chit-Maize: Zea mays class I acidic chitinase Accession number (L16798), Chit-Vitis: Vitis vinifera class IV endochitinase Accession number (U97522), Chit-Chenopod: Chenopodium amaranticolor Chitinase Accession number (D45183), AF112965: Triticum aestivum (bread wheat) beta-1,3-glucanase precursor (Glb3). F. bean Chit-Eg showed 36% identity with published Chitinase sequences in the homology tree (B). The alignment and the phylogeny tree were generated using (DNAMAN V 5.2.9 package, Madison, Wisconsin, USA).

In conclusion plants initiate a complex network of defense mechanisms, among which is a dramatic increase in chitinase activity. For our data we concluded that chitinases are capable of hydrolyzing chitin-containing fungal cell walls and are therefore thought to play a major role in the plant's response. Therefore, one of the strategies used to increase plant tolerance to fungal infection is the constitutive over expression of proteins involved in plant-defense mechanisms (Schickler and Chet, 2004).

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الملخص العربي

عزل جين الكايتينيز المستحث أثناء إصابة الفول البلدي بواسطة فطر *Botrytis fabae*

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تم استخدام أصناف الفول البلدي جيزة ٧١٦ و جيزة ٤٢٩ لتحديد البروتينات المرتبطة بالإصابة المرضية بمرض التبقع الشيكولاتى ، وتم استخدام احد عزلات فطر *Botrytis fabae* المنقى من عينة فول نباتية مجمعة من منطقة النوبارية (محافظة البحيرة، مصر) في عمل تجربة العدوى الاصطناعية. وقد تم عمل التحليل الكمي و النوعي للبروتينات الكلية المعزولة من النباتات المصابة و السليمة و ذلك على فترات زمنية ٨ ، ٢٤ ، ٤٨ ساعة. أوضحت النتائج أن شظية من البروتين وزنها الجزيئى٢٦كيلو دالتون اصبحت اكثر كثافة بعد ٨ ، ٢٤ ،٤٨ ساعة من العدوى وذلك بالنسبة للصنف جيزة٧١٦ . بالإضافة الى ظهور شظية جديدة من البروتين وزنها الجزيئي ٢٩ كيلو دالتون بعد ٢٤ و ٤٨ ساعة و ذلك بالنسبة للصنف جيزة ٧١٦. بالاضافة الى ذلك فى الصنف جيزة ٤٢٩ ظهرت شظية من البروتين ذات وزن الجزيىء ٥٤ كيلودالتون بعد ٨ ، ٢٤ ، ٤٨ ساعة من العدوى و شظيتان ذوات وزن جزيىء ٢٠ و ٢٨ كيلو دالتون بعد ٢٤ ساعة بعد العدوى . أظهر تفاعل النسخ العكسي – و البلمرة المتسلسل أن جين الكايتينيز يتم التعبير عنه في المراحل المبكرة في أوراق الفول البلدي المصابة ، حيث أوضحت النتائج ظهور شظية لجين الكايتينيز ذات وزن جزيئي 900 bp بعد ٨ ، ٢٤ ،٤٨ ساعة من بداية الإصابة المرضية و اختفائها في النباتات السليمة .. و قد تم عمل كلونة لناتج البلمرة المتسلسل داخل ناقل بلازميدى pGEM-T-Easy وقد تم اختيار أربع بلازميدات (متحورة) سميت PNAM1 , PNAM2 , PNAM3 , PNAM4و لتأكيد وجود شظية جين الكيتينيز تم استخدام تفاعل البلمرة المتسلسل و وجد أن البلازميدين PNAM1 , PNAM2 أظهرا نتائج موجبة باستخدام كل من البادئات المتخصصة و الشمولية . .تم عمل تحليل التتابع النيوكليوتيدي للبلازميد PNAM1 وبمقارنته مع تتابعات جينية اخري من بنك الجينات كانت نسبة التشابه ٥٨,١٥%.