Isolation and characterization of Thaumatin-like protein gene from wheat

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ABSTRACT

Fusarium head blight (FHB) or scab, caused by Fusarium graminearum and other Fusarium species is the major disease of wheat in many areas of wheat production in Egypt and throughout the world. Many studies have shown that Thaumatin-like proteins (TLPs) have antifungal activity against FHB disease, therefore,our main objective in this study, is isolating the complete coding sequence of TLP gene from the Egyptian wheat (TLP-Ew) and we choose Egyptian wheat variety (Sakha 61) to carry out this investigation. We succeeded to isolate and amplify the full length of the target gene using specific primers that have been designed by our Bioinformatic Center. DNA sequence comparison was carried out using Genbank database and the sequence of the TLP-Ew gene. This comparison gave 100 % similarity between TLP-Ew gene and Triticum aestivum Thaumatin-like protein mRNA with the GenBank accession no. AF384146. No other TLP genes could be isolated from other wheat varieties. Amplification of the isolated TLP gene gave a complete coding sequence, representing a DNA fragment of 522 bp and a protein of 173 amino acids.

Keywords: *Isolation, Thaumatin-like protein gene, Egyptian wheat.*

INTRODUCTION

Wheat is a member of the family *Poaceae* (formerly *Gramineae*), which includes the major cereal crops of the world such as maize, wheat and rice. Among the food crops, wheat (hexaploid type) is one of the most abundant source of energy and proteins for the world population. Ninety percent of wheat grown today is used for the preparation of bread and other baked produets.

Hexaploid wheat (*Triticum aestivum* L.) was originated about 8000 years ago from the hybridization of tetraploid wheat with diploid *Aegilops tauschii* Coss, containing the D-genome. Thus, the bread wheat D-genome is evolutionary young and shows a low degree of polymorphism in the bread wheat gene pool (Bossolini *et al.*, 2006). Wheat like any plant is continually exposed to pathogen attack. Therefore, plants have evolved a wide array of defences mechanisms against pathogen attack (Hammond-Kosack and Jones,1996). These response mechanisms are hypersensitive response (HR), reactive oxygen species (ROS) and oxidative enzymes, cell wall modification, phytoalexins, systemic acquired resistance (SAR) and pathogenesis related proteins (PR-proteins) (Dangl and Jones, 2001; Heath,

2000; Morris, 2001; Romeis, 2001; Takken and Joosten, 2000). Among the most frequently observed biochemical events that follow plant infection by pathogens is the induction and accumulation of pathogenesis related protein families (PR-proteins). The PR proteins are classified into 17 families based on the sequence similarities, serologic or immunologic relationships and enzymatic properties (Van Loon *et al.*, 2006). One of these groups, the PR-5 family, comprises unique proteins with diverse functions. Therefore, TLPs can be classified into three sub-groups, (i) those produced in response to pathogen infection, (ii) those produced in response to osmotic stress, also called osmotins, and (iii) antifungal proteins present in cereal grains. (Breiteneder, 2004). Thaumatin like proteins are similar to the intensely sweet tasting protein, Thaumatin from the West African shrub *Thaumatococcus daniellii* fruits, even though, none of them have been found to have a sweet taste (Ghosh and Chakrabarti, 2005).

The TLPs are generally resistant to proteases, pH, and heat induced denaturation. This is due to the presence of 16 conserved cysteines that form eight disulfide bridges (Caroline *et al.*, 2007). Multiple sequence alignment analysis revealed that the TLPs in general have16 cysteine residues found in barley, tomato, potato and oat. In other cases such as wheat and barley the TLPs have only 10 cysteine residues (Campos *et al.*, 2002). TLP and several other PR-5 proteins have antifungi activity, whereas the Thaumatin protein does not. (Malehorn *et al.*, 1994). According to Fierens *et al.* (2008) Thaumatin-like protein is a membrane permeabilizing protein. Moreover, TLPs have been shown to have inhibitory effects on hyphal growth and spore of many fungi *in vitro* because, they found that the TLPs bind to insoluble β-1,3-glucan and have an endo- β -1,3-glucanase activity (Selitrennikoff, 2001). This activity has been observed against several pathogenic and non-pathogenic fungi (Campos *et al.*, 2002). Among the pathogenic fungi which the TLP effects on, the *Fusarium graminearum* infecting the wheat plant causing the fusarium head blight (FHB) disease (Sutton, 1982; McMullen *et al.*, 1997). Between 1993 and 2001, in the United States, an estimated US$ 8 billion loss was incurred from FHB (Caroline *et al.*, 2007). The aim of this work was to design specific primers for isolation of the complete coding sequence of Thaumatin-like protein gene from the Egyptian wheat.

MATERIALS AND METHODS

This investigation was carried out in the Plant Biotechnology Research Laboratory, Faculty of Agriculture, Cairo University, Giza, Egypt, and in the Gene analysis unit, VACSERA holding company, Agouza, Giza, Egypt.

Plant materials

The grains of *Triticum aestivum* L. cv. SAKHA 61 are obtained from the Agricultural Research Centre, Crops Research Institute, ARC, Giza.

Genomic DNA extraction and purification

Extraction of total DNA was performed using CTAB protocol according to Doyle and Doyle 1990 (Sambrook *et al.*, 1989).

Amplification of Thaumatin-like protein gene (PCR)

The PCR amplification was carried out using 200 ng of total genomic DNA as a template and Go Taq DNA polymerase using two groups of oligonucleotides. Primers design were performed using DNASTAR suite of lasergene programs (DNASTAR, Inc., Madison, USA).

First group

The sequences of the primers used in the nested PCR are:

1-F 5`- GC GT GAATTC CT TCC TCC TCC TCG CTG TTT- 3`

1-R 5`- GCGT GAATTC AG TCC ATG GCA AGG TTG AAG -3`

Second group

Another primers were synthesized for thaumatin like protein (*TLP*) gene amplification. Then the recognition site for *BgLII* and *EcoRI* were added to the primer sequence of F-Exp and R-Exp. respectively with the the following sequences.

F-EXP **5`-AA AGATCT ATG GCG ACC TCG CGG TGC T-3`**

R-EXP **5`-GG GAATTC TCA TGG GCA GAA GGT GAT CTG GTA G -3`**

DNA amplifications were performed in a thermal cycler using initial denaturation at 94 °C for 5 min. followed by 30 cycles of 1 min at 95 °C, 20 sec at 61 °C and 45 sec at 72 °C. One additional complete extension cycle was performed for 7 min at 72 °C. The product ranged in sizes from 300-400 bp in nested PCR and from 500-600 bp in the seconed PCR. The amplified products were then analyzed on 1 % (w/v) agarose gel and visualized by ethidium bromide staining. The procducts were performed according to the manufacturers specifications (Promega, USA, 1991).

Purification of DNA from agarose gels

The bands of interest were excised from the gel using a sterile scalpel blade and put in 1.5 ml Eppendorf tube. The DNAs were purified from the agarose gel according to the instructions in the QIAquick Spin Handbook.

Cloning of PCR product

The TLP amplified fragment was cloned into pUC19 vector containing the recognition site of *EcoRI* in it's 5` and *BamH I* in its 3` to enable the ligation of the target gene, containing the recognition site of *BgLII* in its 5` and *EcoRI* in its 3`. This ligation mixture was transformed into *E.coli Top10* strain.

Transformation protocol

The ligation reaction was transformed into *E.coli* as the domestic host for that purpose.Competent cells of TOP10 were prepared for transformation of recombinant plasmid according to the method of Sambrook *et al.* (1989). The detection of the recombinant plasmid was done by blue/white colony screening.

Isolation and purification of recombinant plasmid DNA from transformed cells

Minipreparation of plasmid DNA and its recombinant derivatives were purified using Wizared plus SV minipreps DNA purification system kit. (Cat.# A1330). To confirm the cloning steps and to identify each clone, purified plasmid DNA was used as template by using the same primer pairs used befor, and the resulting product was analyzed along side the original total-community PCR products.

Sequencing of the cloned gene

The dideoxyribonucleoside chain termination procedure originally developed by Sanger *et al.* (1977) was employed for sequencing the double-stranded recombinant DNA plasmid obtained during the cloning procedure. The DNA sequence was determined by automated DNA sequencing method using ABI PRISM Big Dye Terminator V3L Cycle sequencing Kit (Applied Biosystems, USA) in conjuncation with ABI PRISM 310 Genetic Analyzer. Cycle sequencing was performed using the HVD Thermal Cycler, and the reaction was conducted in a total volume of 20 µl, containing 4 µl of Big Dye

Terminator, 4 µl of 5× Cycle sequencing buffer, 1 µg of plasmid DNA and 3.2 pmol of M13 universal forward primer (provided with the kit). The cycle sequencing program was 94 °C for 1 min, 65 °C for 20 sec and 72 °C for 45 sec repeated for 30 cycles. The nucleotide sequencing was determined automatically by the electrophoresis of the cycle sequencing reaction product on 310 Genetic Analyzer. Alignment of DNA sequences was performed using the BLST comparison with GenBank.

RESULTS AND DISCUSSION

Extraction and purification of genomic DNA from leaves of wheat plants

Leaves of wheat plants (Sakha 61) were collected from an open field of the Plant Biotechnology Research Laboratory, Faculty of Agriculture, Cairo University, Giza, Egypt., to extract and purify genomic DNA. Genomic DNA from wheat leaves was extracted by using CTAB protocol according to (Sambrook *et al.*, 1989). This procedure allows up to 100µg a highly purified Genomic DNA isolated as shown in Fig.(1).

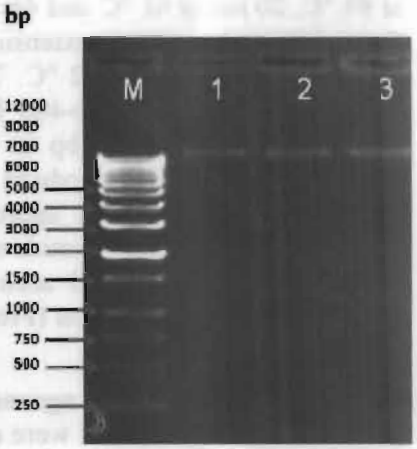

Fig. (1): Isolation of genomic DNA from the leaves of the wheat (Sakha 61) (1% agarose gel.) M: 1Kb DNA ladder (STRATAGENE CAT. # 201115-81). Lanes 1,2 and 3: The isolated genomic DNA from the leaf.

Amplification of the Thaumatin- like protein gene

According to our knowledge, there is no previous sequence for *TLP* gene from wheat (SAKHA 61). Therefore, multiple sequence alignment for the coding sequences of six *TLP* genes from different varieties of *Triticum aestivum L.* (bread wheat) were used for designing specific primers for *TLP* genes by using ExPASy Database. Two of these genes were isolated from genomic DNA with the GenBank accessions NO. AF389884 and AF389883. The others were isolated from mRNA with the GenBank accessions NO. X58394, X97687, AF442967 and AF384146 (Rebmann *et al.*, 1991; Mingeot and Jacquemin, 1997; Kuwabaraa *et al.*, 2002).

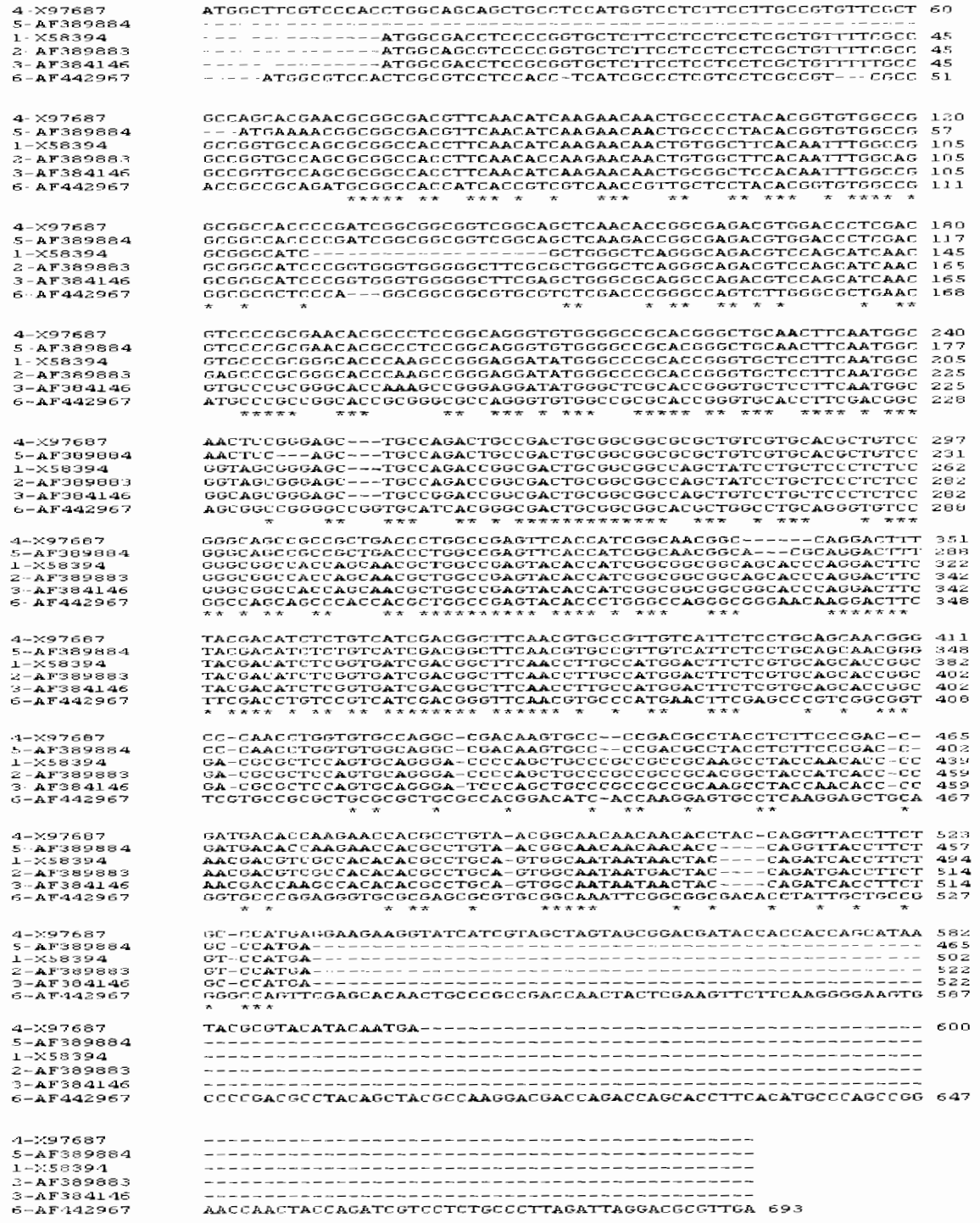

Fig. (2): Alignment between six TLP genes from Triticum aestivum.

Three highly conserved sequences were selected for multiple sequence alignment X58394 (Rebmann *et al.*, 1991) and AF384146 isolated from mRNA and AF389883 isolated from genomic DNA by using ExPASy Database as shown in Fig. (3). to amplify the open reading frames of our target gene.

```
1-X58394        ATGGCGACCTCCCCGGTGCTCTTCCTCCTCCTCGCTGTTTTCGCCGCCGGTGCCAGCGCG 60
2-AF389883      ATGGCAGCGTCCCCGGTGCTCTTCCTCCTCCTCGCTGTTTTCGCCGCCGGTGCCAGCGCG 60
3-AF384146      ATGGCGACCTCCGCGGTGCTCTTCCTCCTCCTCGCTGTTTTTGCCGCCGGTGCCAGCGCG 60
                ***** * *** ****************************** ******************

1-X58394        GCCACCTTCAACATCAAGAACAACTGTGGCTTCACAATTTGGCCGGCGGGCATC------ 114
2-AF389883      GCCACCTTCAACACCAAGAACAACTGTGGCTTCACAATTTGGCAGGCGGGCATCCCGGTG 120
3-AF384146      GCCACCTTCAACATCAAGAACAACTGCGGCTCCACAATTTGGCCGGCGGGCATCCCGGTG 120
                ************** ************ **** *********** **********

1-X58394        --------------GCTGGGCTCAGGGCAGACGTCCAGCATCAACGTGCCCGCGGGCACC 160
2-AF389883      GGTGGGGGCTTCGCGCTGGGCTCAGGGCAGACGTCCAGCATCAACGAGCCCGCGGGCACC 180
3-AF384146      GGTGGGGGCTTCGAGCTGGGCGCAGGCCAGACGTCCAGCATCAACGTGCCCGCGGGCACC 180
                              ******* **** ******************** *************

1-X58394        CAAGCCGGGAGGATATGGGCCCGCACCGGGTGCTCCTTCAATGGCGGTAGCGGGAGCTGC 220
2-AF389883      CAAGCCGGGAGGATATGGGCCCGCACCGGGTGCTCCTTCAATGGCGGTAGCGGGAGCTGC 240
3-AF384146      AAAGCCGGGAGGATATGGGCTCGCACCGGGTGCTCCTTCAATGGCGGCAGCGGGAGCTGC 240
                 ******************* ************************** ************

1-X58394        CAGACCGGCGACTGCGGCGGCCAGCTATCCTGCTCCCTCTCCGGGCGGCCACCAGCAACG 280
2-AF389883      CAGACCGGCGACTGCGGCGGCCAGCTATCCTGCTCCCTCTCCGGGCGGCCACCAGCAACG 300
3-AF384146      CGGACCGGCGACTGCGGCGGCCAGCTGTCCTGCTCCCTCTCCGGGCGGCCACCAGCAACG 300
                * ************************ ************************************

1-X58394        CTGGCCGAGTACACCATCGGCGGCGGCAGCACCCAGGACTTCTACGACATCTCGGTGATC 340
2-AF389883      CTGGCCGAGTACACCATCGGCGGCGGCAGCACCCAGGACTTCTACGACATCTCGGTGATC 360
3-AF384146      CTGGCCGAGTACACCATCGGCGGCGGCGGCACCCAGGACTTCTACGACATCTCGGTGATC 360
                *************************** ********************************

1-X58394        GACGGCTTCAACCTTGCCATGGACTTCTCGTGCAGCACCGGCGACGCGCTCCAGTGCAGG 400
2-AF389883      GACGGCTTCAACCTTGCCATGGACTTCTCGTGCAGCACCGGCGACGCGCTCCAGTGCAGG 420
3-AF384146      GACGGCTTCAACCTTGCCATGGACTTCTCGTGCAGCACCGGCGACGCGCTCCAGTGCAGG 420
                ************************************************************

1-X58394        GACCCCAGCTGCCCGCCGCCGCAAGCCTACCAACACCCCAACGACGTCGCCACACACGCC 460
2-AF389883      GACCCCAGCTGCCCGCCGCCGCACGGCTACCATCACCCCAACGACGTCGCCACACACGCC 480
3-AF384146      GATCCCAGCTGCCCGCCGCCGCAAGCCTACCAACACCCCAACGACCAAGCCACACACGCC 480
                ** ******************** * ****** ************   *************

1-X58394        TGCAGTGGCAATAATAACTACCAGATCACCTTCTGTCCATGA 502
2-AF389883      TGCAGTGGCAATAATGACTACCAGATGACCTTCTGTCCATGA 522
3-AF384146      TGCAGTGGCAATAATAACTACCAGATCACCTTCTGCCCATGA 522
                *************** ********** ******** ******
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***Fig. (3): Alignment between the three TLP genes from Triticum aestivum* L.**

From this alignment it can be deduced that this *TLP* gene isolated from wheat genome has no introns in it, whereas the sequence of the *TLP* gene isolated from the Genomic DNA (GenBank accession NO. AF389883) is complementary to that of *TLP* genes from the mRNA (GenBank accessions NO. X58394 and AF384146. Also the PR-S genes isolated from tobacco do not contain introns (Van Kan *et al.*, 1989). However, the *TLP* gene from *Lentinula edodes* contains 12 introns (Sakamoto *et al.*, 2006).

From the previous alignment , five primers were chosen and only primer number one (1-F and 1-R) is working, but the other four primers gave unspecific bands. PCR amplification of the TLP fragment was carried out using 1-F and 1-R primers to amplify partial sequense of *TLP* gene ~ 300bp Fig.(4). TLP fragment (~ 300bp) was purified from the gel by Gel Extraction Kit (QIAquick Gel Extraction Kit (50) Cat. No. 28704 QIAGEN.

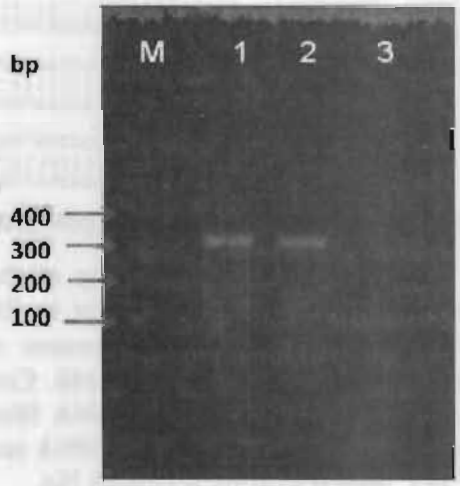

Fig. (4): The PCR amplification of TLP by 1-F and 1-R primers, (2% agarose gel). M: 100bp low ladder (Sigma P-1473). Lanes 1 and 2: TLP fragment ~ 300bp. Lane 3: negative control.

The incomplete nucleotide sequence of the TLP fragment was determined using automated sequencer (ABI PRISM 310 Genetic Analyzer) and the sequence was 356 bp with 118 amino acids (Fig. 5).

```
GCCGCCGGTGCCAGCGCGGCCACCTTCACATCAAGAACAACTGTCGGCTNCACAATTTGG    62
A   A   G   A   S   A   A   T   F   T   S   R   T   T   V   G   X   T   I   W

CCGGCGGGCATCCCGGTGCGTGGGGGCTTCGNGCTGGGCNCAGGNCAGACGTCCAGCATC   122
P   A   G   I   P   V   G   G   G   F   X   L   G   X   X   Q   T   S   S   I

AACGTGCCCGCCGGCAACCNAAGCCGGGAGGATATGGGCNCGCACCGGGTGCTCCTTCAAT   182
N   V   P   A   G   T   X   A   G   R   I   W   X   R   T   G   C   S   F   N

GGCGGNAGCGGGAGCTGCCNGACCGGCGACTGCGGCGGCCAGCTNTCGTGCTCCCTGTCC   242
G   X   S   G   S   C   X   T   G   D   C   G   G   Q   X   S   C   S   L   S

GGGCGGCCACCAGCAACGCTGGCCGAGTACACCATCGGCGGCGGCNGCACCCAGGACTTC   302
G   R   P   P   A   T   L   A   E   Y   T   I   G   G   G   X   T   Q   D   F

TACGACATCTCGGTGATCGACGGCTTCAACCUGGCATGGATTGGAATTCACGCA         356
Y   D   I   S   V   I   D   G   F   N   X   A   W   I   G   I   H   A
```

Fig. (5): The nucleotide sequence of the incomplete fragment of the TLP. The amino acid sequence translated from the open reading frame is given in the single-letter code below the nucleotide sequence generated on ExPASy Database (356 bp and 118 a.a).

```
gb|AF384146.1|AF384146  Triticum aestivum thaumatin-like protein mRNA, complete cds
Length=663

 GENE ID: 543342 Ta-TLP | thaumatin-like protein [Triticum aestivum]

 Score =  608 bits (329),  Expect = 5e-171
 Identities = 338/342 (98%), Gaps = 2/342 (0%)
 Strand=Plus/Plus

Query  3    GCCGCCGGTGCCAGCGCGGCCACCTTC-ACATCAAGAACAACTGTCGGCTCCACAATTTG  61
            ||||||||||||||||||||||||||| |||||||||||||| ||||||||||||||||
Sbjct  54   GCCGCCGGTGCCAGCGCGGCCACCTTCAACATCAAGAACAACTG-CGGCTCCACAATTTG  112

Query  62   GCCGGCGGGCATCCCGGTGGGTGGGGGCTTCGAGCTGGGCGCAGGGCAGACGTCCAGCAT  121
            ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  113  GCCGGCGGGCATCCCGGTGGGTGGGGGCTTCGAGCTGGGCGCAGGCCAGACGTCCAGCAT  172

Query  122  CAACGTGCCCGCGGGCACCAAAGCCGGGAGGATATGGGCTCGCACCGGGTGCTCCTTCAA  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  173  CAACGTGCCCGCGGGCACCAAAGCCGGGAGGATATGGGCTCGCACCGGGTGCTCCTTCAA  232

Query  182  TGGCGGCAGCGGGAGCTGCCGGACCGGCGACTGCGGCGGCCAGCTGTCCTGCTCCCTCTC  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  233  TGGCGGCAGCGGGAGCTGCCGGACCGGCGACTGCGGCGGCCAGCTGTCCTGCTCCCTCTC  292

Query  242  CGGGCGGCCACCAGCAACGCTGGCCGAGTACACCATCGGCGGCGGCGGCACCCAGGACTT  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  293  CGGGCGGCCACCAGCAACGCTGGCCGAGTACACCATCGGCGGCGGCGGCACCCAGGACTT  352

Query  302  CTACGACATCTCGGTGATCGACGGCTTCAACCTTGGCATGGA  343
            |||||||||||||||||||||||||||||| ||||||
Sbjct  353  CTACGACATCTCGGTGATCGACGGCTTCAAGCTTGCCATGGA  394
```

Fig. (6): The Blast of the TLP gene fragment on the GenBank.

The sequence of 356 bp of the *TLP* gene fragment was 95% similar to *Triticum aestivum* L. thaumatin-like protein mRNA with the accession NO. AF384146. Computer analysis was done using the DNA Blast web site (Fig. 6). According to the DNA sequence of *TLP* gene (Genbank accession No. AF384146), the forward and reverse oligonucleotide primers. F-Exp and R-Exp, were designed for amplification of the complete coding sequence of *TLP* gene from the genomic DNA of Sakha 61. Fig. (7) shows the position of F-Exp and R-Exp primers.

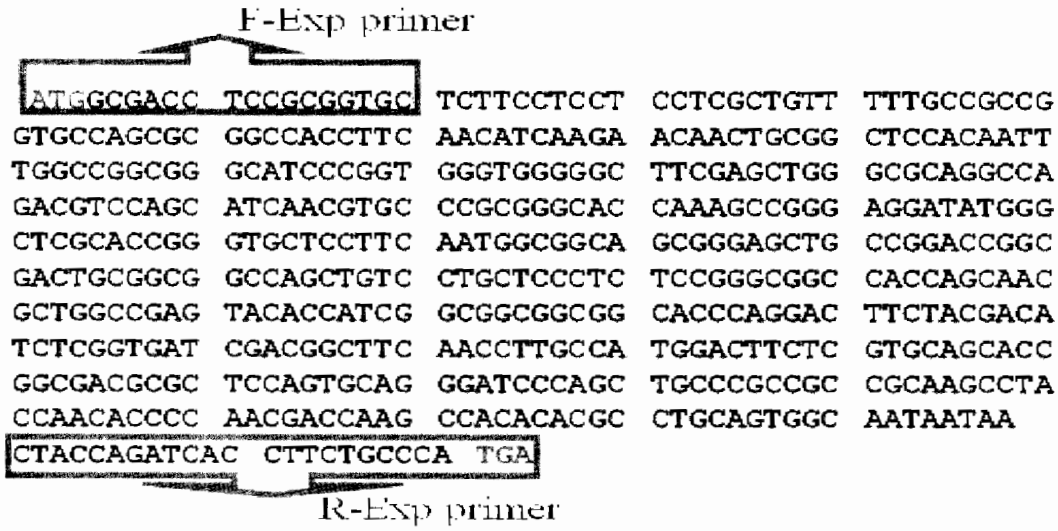

Fig. (7): The position of F-Exp and R-Exp primers on the AF384146 sequence.

PCR amplification of the *TLP* gene was carried out using F-Exp and R-Exp primers to amplify the complete coding sequence of the gene which represent a fragment of ~ 500 bp. Fig. (8) shows that the size of the amplifed product is near to the expected size of the gene. This PCR product flanked a start codon and stop codon. Then the PCR product was purified by Gel Extraction kit (QIAquick Gel Extraction Kit (50) Cat. No. 28704 QIAGEN) to be ready for ligation reaction.

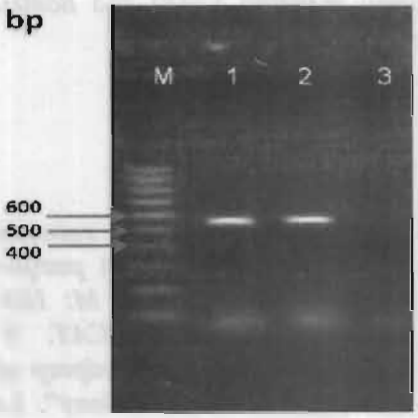

Fig. (8): The PCR amplification of TLP (2% agarose gel). M: 100 bp low ladder (Sigma). Lanes 1 and 2: PCR products. Lane 3: negative control.

Rebmann (1991) sequenced wheat cDNA (GenBank accession NO. X58394) encoding for Thaumatin-like protein isolated from wheat *(Triticum aestivum* L.) inoculated with *Erysiphe graminis* f. sp. *Horde.* The ORF of the cDNA contains 502 bp. However, Mingeot and Jacquemin in (1997) have observed that the gbx3832, a wheat cDNA encoding for *TLP* gene, (GenBank accession NO. X97687) contains an ORF 600bp. whereas Kuwabaraa (2002) has characterized a cDNA of WAS-3a encoding for TLPs from winter wheat cells (GenBank accession NO. AF442967), with ORF of 693 bp.

Cloning of the Thaumatin-like protein (TLP)

a) Ligation of TLP

The *TLP* gene was cloned into pUC19 vector which contains the recognition site of *EcoRI* in its 5` and *BamHI* in its 3` to enable the ligation of the target gene, which contains the recognition site of *BgLII* in its 5` and *EcoRI* in its 3`. (Fig. 9). This ligation mixture was transformed into *E.coli* Top10 strain.

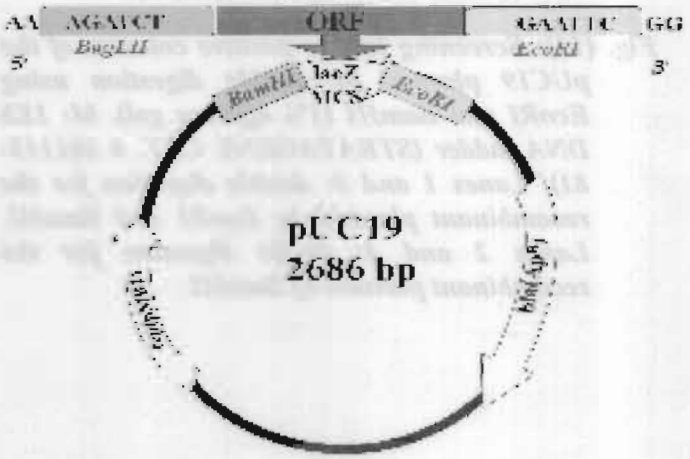

Fig. (9): The restriction map of the pUC19 with the TLP.

b) Screening for the recombinant plasmid

Screening for the recombinant plasmid was done by using a variety of molecular biology techniques such as blue and white colony, screening with minipreps DNA purification method, double restriction digestion using *EcoRI* and *BamHI* and PCR experiment.

c) Minipreps DNA purification method

The plasmids of the selected white clones and some blue clones were isolated using Wizared plus SV minipreps DNA purification system (Cat.# A1330). After screening with minipreps DNA purification method, the positive colonies had higher molecular weight than the negative colonies as shown in Fig. (10).

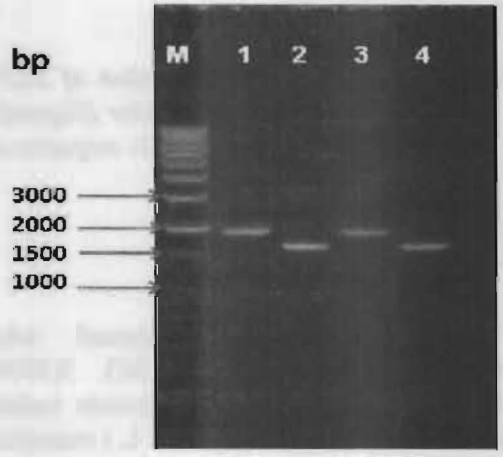

Fig. (10): Screening for recombinant plasmid by minipreps DNA purification method (1% agarose gel). M: 1Kb DNA ladder (STRATAGENE CAT. # 201115-81). Lanes 1 and 3: miniprep of recombinant plasmid "white colony". Lanes 2 and 4: miniprep of pUC19 "blue colony".

d) Double restriction digestion using *EcoRI* and *BamHI*

The insert was checked by electrophoresis on 1% agarose gel after double restriction digestion with *EcoRI* Promega (cat. # R6011) and *BamHI* Promega (cat. # R602) as shown in Fig.(11). Results of double digestion indicated the electrophoretic mobility of the *TLP* fragment of ~ 500 bp associated with a higher molecular weight band of about 2700 bp refereed to pUC19 linear plasmid lanes 1and 3.

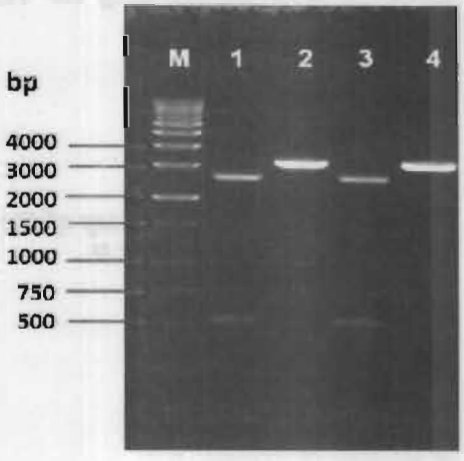

Fig. (11): Screening for the positive colonies of the pUC19 plasmid by double digestion using EcoRI and BamHI (1% agarose gel). M: 1Kb DNA ladder (STRATAGENE CAT. # 201115-81). Lanes 1 and 3: double digestion for the recombinant plasmid by EcoRI and BamHI. Lanes 2 and 4: single digestion for the recombinant plasmid by BamHI.

h) PCR experiment detection.

PCR amplification detection of the recombinant plasmid carrying the TLP fragment using F-Exp and R-Exp primers was carried out with the DNA of recombinant plasmid as a confirmation step, and examined by gel electrophoresis Fig. (12).

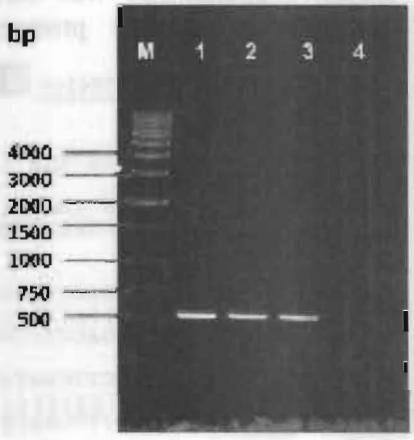

Fig .(12): Screening for the positive colonies of the PUC19 plasmid by PCR (1% agarose gel). M: 1Kb DNA ladder. (STRATAGENE CAT. # 201115-81). Lanes 1,2 and 3: positive colonies of the PUC19 plasmid. Lane 4: negative colony.

Sequence of the complete *TLP* gene

The complete *TLP* gene was sequenced using the m13 forward primer.Sequence determination was carried out using the dideoxynucleotide method, using fluorescent bases and analyzed on an automated Applied Biosystems Model 310 sequencer and the sequence was as in Fig. (13).

```
atggcgacctccgcggtgctcttcctcctcctcgctgtttttgccgccggtgccagcgcg
 M  A  T  S  A  V  L  F  L  L  L  A  V  F  A  A  G  A  S  A
gccaccttcaacatcaagaacaactgcggctccacaatttggccggcgggcatcccggtg
 A  T  F  N  I  K  N  N  C  G  S  T  I  W  P  A  G  I  P  V
ggtgggggcttcgagctgggcgcaggccagacgtccagcatcaacgtgcccgcgggcacc
 G  G  G  F  E  L  G  A  G  Q  T  S  S  I  N  V  P  A  G  T
aaagccgggaggatatgggctcgcaccgggtgctccttcaatggcggcagcgggagctgc
 K  A  G  R  I  W  A  R  T  G  C  S  F  N  G  G  S  G  S  C
cggaccggcgactgcggcggccagctgtcctgctccctctccgggcggccaccagcaacg
 R  T  G  D  C  G  G  Q  L  S  C  S  L  S  G  R  P  P  A  T
ctggccgagtacaccatcggcggcggcggcacccaggacttctacgacatctcggtgatc
 L  A  E  Y  T  I  G  G  G  G  T  Q  D  F  Y  D  I  S  V  I
gacggcttcaaccttgccatggacttctcgtgcagcaccggcgacgcgctccagtgcagg
 D  G  F  N  L  A  M  D  F  S  C  S  T  G  D  A  L  Q  C  R
gatcccagctgcccgccgccgcaagcctaccaacaccccaacgaccaagccacacagccc
 D  P  S  C  P  P  P  Q  A  Y  Q  H  P  N  D  Q  A  T  H  A
tgcagtggcaataataactaccagatcaccttctgcccatga
 C  S  G  N  N  N  Y  Q  I  T  F  C  P  -
```

Fig. (13): The DNA sequence of the TLP gene isolated from Egyptian wheat and translation of nucleotide sequence generated on ExPASy Database (522 bp and 173 a.a).

DNA sequence was edited to remove any primer or vector sequence associated with the sequence using the EditSeq module Lasergene suite program ver.7.0 (DNA Star inc). DNA sequence comparison was carried out with those entries in the primary data base. Genbank at (National Center for Biotechnology Information (NCBI). The sequence of the *TLP* gene fragment was 100% similar to *Triticum aestivum* Thaumatin-like protein mRNA with the accession number AF384146 Fig. (14).

```
>gb|AF384146.1|AF384146 Triticum aestivum thaumatin-like protein mRNA, complete cds
Length=663

GENE ID: 50 31, Ta TLP | thaumatin-like protein [Triticum aestivum]

Score =  965 bits (522),  Expect = 0.0
Identities = 522/522 (100%), Gaps = 0/522 (0%)
Strand=Plus/Plus

Query  9    ATGGCGACCTCCGCGGTGCTCTTCCTCCTCCTCGCTGTTTTTGCCGCCGGTGCCAGCGCG  68
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12   ATGGCGACCTCCGCGGTGCTCTTCCTCCTCCTCGCTGTTTTTGCCGCCGGTGCCAGCGCG  71

Query  69   GCCACCTTCAACATCAAGAACAACTGCGGCTCCACAATTTGGCCGGCGGGCATCCCGGTG  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72   GCCACCTTCAACATCAAGAACAACTGCGGCTCCACAATTTGGCCGGCGGGCATCCCGGTG  131

Query  129  GGTGGGGGCTTCGAGCTGGGCGCAGGCCAGACGTCCAGCATCAACGTGCCCGCGGGCACC  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  132  GGTGGGGGCTTCGAGCTGGGCGCAGGCCAGACGTCCAGCATCAACGTGCCCGCGGGCACC  191

Query  189  AAAGCCGGGAGGATATGGGCTCGCACCGGGTGCTCCTTCAATGGCGGCAGCGGGAGCTGC  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  192  AAAGCCGGGAGGATATGGGCTCGCACCGGGTGCTCCTTCAATGGCGGCAGCGGGAGCTGC  251

Query  249  CGGACCGGCGACTGCGGCGGCCAGCTGTCCTGCTCCCTCTCCGGGCGGCCACCAGCAACG  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252  CGGACCGGCGACTGCGGCGGCCAGCTGTCCTGCTCCCTCTCCGGGCGGCCACCAGCAACG  311

Query  309  CTGGCCGAGTACACCATCGGCGGCGGCGGCACCCAGGACTTCTACGACATCTCGGTGATC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  312  CTGGCCGAGTACACCATCGGCGGCGGCGGCACCCAGGACTTCTACGACATCTCGGTGATC  371

Query  369  GACGGCTTCAACCTTGCCATGGACTTCTCGTGCAGCACCGGCGACGCGCTCCAGTGCAGG  428
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  372  GACGGCTTCAACCTTGCCATGGACTTCTCGTGCAGCACCGGCGACGCGCTCCAGTGCAGG  431

Query  429  GATCCCAGCTGCCCGCCGCCGCAAGCCTACCAACACCCCAACGACCAAGCCACACACGCC  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  432  GATCCCAGCTGCCCGCCGCCGCAAGCCTACCAACACCCCAACGACCAAGCCACACACGCC  491

Query  489  TGCAGTGGCAATAATAACTACCAGATCACCTTCTGCCCATGA  530
            ||||||||||||||||||||||||||||||||||||||||||
Sbjct  492  TGCAGTGGCAATAATAACTACCAGATCACCTTCTGCCCATGA  533
```

Fig. (14): The result of the Blast of the complete TLP gene on the GenBank.

Amino acid sequences of wheat and its homologues were aligned using CLUSTAL W. In the amino acids sequence of the TLP isolated from wheat leaves, which have the accessions NO. AF389883, AF384146 and the recombinant *TLP* gene which was isolated from genomic DNA (Sakha 61). 10 cysteine can be observed and the 16 cysteine in the amino acids sequence of the oat, barley and tomato (Fig. 15).

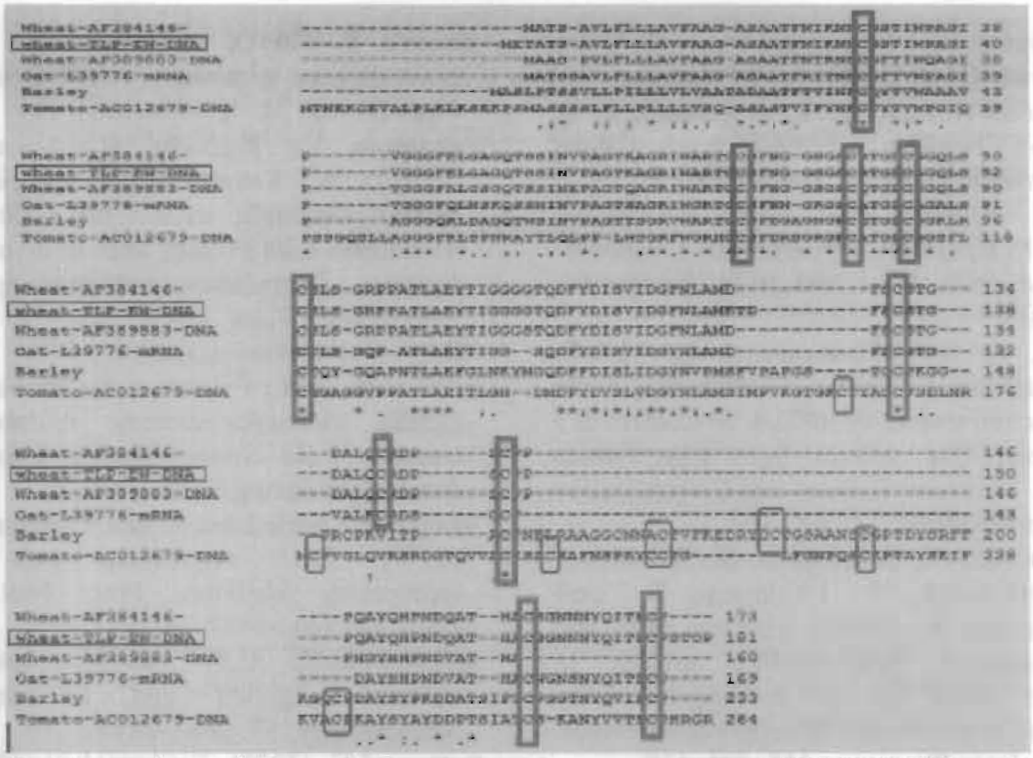

Fig. (15): Comparison of N-terminal amino acid sequences of purified TLP-EW protein with several TL proteins. Amino acid residues, which are conserved in all thaumatin-like proteins, are marked with asterisks. Positions of the conserved cysteine residues are boxed.

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الملخص العربي

إستخدام تقنيات الهندسة الوراثية فى عزل وتوصيف جين Thaumatin-like protein من نبات القمح

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يعتبر مرض لفحة السنابل Fusarium head blight (FHB) المتسبب نتيجة الاصابة بفطر *Fusarium graminearum* وغيره من فطريات ال Fusarium , المرض الرئيسى الذى يصيب نبات القمح فى العالم. ولقد بينت العديد من الدراسات التى أجريت أن ال Thaumatin-like proteins لها فعل مضاد للفطريات الممرضة و منها مرض لفحة السنابل FHB و فى هذه الدراسة تم عزل و توصيف - على المستوى الجزئ- أحد الجينات من صنف القمح المصرى سخا-٦١ و الذى رمز له بـ*TLP-Ew*.ولتجسيم (Amplification) التتابع الشفرى الكامل لهذا الجين من القمح المصرى ,تم تصميم بادئات متخصصة و محددة و قد تم الاستعانة بعمل مقارنات تتابعات الـ DNA ببنك المعلومات المحفوظة فى بنك الجينات الدولى (Database Genbank) و بينت النتائج التى تم الحصول عليها من الدراسة الحالية تشابها بنسبة ١٠٠% لتتابع الجين TLP المحفوظ فى قاعدة بيانات البنك الدولى (Genbank accession No.AF384146) و ذلك المخلق من القمح *Triticum activum* Thaumatin-like protein gene. و لقد بينت الدراسة الحالية عدم إمكانية عزل أية جينات خاصة بـ Thaumatin-like protein من اصناف القمح المصرية الاخرى و أظهرت نتائج تجسيم (Amplification) الجين *TLP-Ew* المعزول من القمح المصرى سخا-٦١ تتابع شفرى كامل لمقطع DNA و قدره 522bp و بروتين قدره 174 حمض امينى.