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Phylogenetic characterization and antifungal activity of recombinant defensin protein from *Triticum aestivum*

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Abstract

Defensins protein plays an important role in innate immune defense against infectious diseases in animals and plants. In our study and for the first time, common wheat (*Triticum aestivum*) defensin gene was fully characterized. The protein encodes from a signal peptide region of 25 amino acids. Homology searches showed that *T. aestivum* defensin have a highest identity (72-64 %) with other defensin selected sequences. A multiple sequence alignment indicates very well highly conserved regions include eight cystiene residues, α-helix, loop, and β-sheet. A phylogenetic analysis of the *T. aestivum* defensin gene sequence among other plant defensin sequences further confirmed that the *T. aestivum* sequence is very closely related to *Triticum durum* defensin sequences, and thus, is likely to have the same expressed structure and function. Moreover, the recombinant defensin protein was expressed *in vitro* and it show a strong antifungal activity against pathogenic strain *Puccinia striiformis*. Our study indicate that recombinant defensin protein may be a powerful tool for common wheat treatment.

Introduction

Wheat is one of the important crops that people need, to provide them with their daily bread (Shewry, 2009).

World wheat production increased dramatically (CIMMYT, 1996) . In Iraq the wheat production was expected to be1.75 million tons last year, and this is expected to rise to 1.85 million tons this year, an increase of only 5.4 percent (http://www.iraq-businessnews.com).

Several diseases particularly rust and smuts have drastically decreased grain yield and quality of wheat (Byerlee and Dubin, 2009). A new, particularly virulent strain of *Puccinia striiformis*, the fungus that causes wheat yellow rust, surfaced in the Middle East in 2010. The fungus has wiped out between 20 to 50% of Syria's wheat yield in 2010. It has also spread to neighboring Lebanon, Iran, Iraq and Turkey (Yahia, 2011). Therefore its yield losses up to 10 % were recorded in most wheat fields (Salman *et al.*, 2006).

Several important genes play an important role in innate immune system, their proteins or peptides have strongest antibacterial and antifungal activity, Defensin is one of them (Hellgren and Ekblom, 2010).
Defensins are small cysteine - rich cationic proteins found in both vertebrates and invertebrates. They have also been reported in plants (Kerkis *et al.*, 2014). They are active against bacteria, fungi and many enveloped and non-enveloped viruses . They consist of 18 – 45 amino acids including six to eight conserved cysteine residues. cells of the immune system contain these peptides to assist in killing phagocytized bacteria. Most defensins function by binding to the microbial cell

membrane and, once embedded, forming pore – like membrane defects that allow efflux of essential ions and nutrients (Tran *et al*., 2008).

The Primary structure of the defensin have been discussed thoroughly. All are cationic with arginine as the predominant cationic resides, and all are stabilized by three disulfide bridges, the mature of α – defensin (classic defensin) contain 29 – 35 amino acid the β- defensin 38 – 42 , and the insect defensin 29 – 34 (Ganz and Lehrer,1994), (Hoffman and Hetru,1992) .

Defensin has been shown to express in various plant tissues. Resistance of tobacco plants to the fungal leaf pathogen *Alternaria longipes* was highly activated during expression of defensin (Lee *et al.*, 2008) and similarly in tomato to *Alternaria solani* (Parashina *et al.*, 2000). Moreover defensin showed to protect pea against blackleg disease (Hiruma *et al.*, 2011). It was also shown that the expression of defensin in potato provided resistance against fungus *Verticillium dahliae* (Gao *et al.*, 2000).

In this study a new defensin gene sequence from *T. aestivum* has been cloned, characterized, and expressed. The purified recombinant protein has been tested *in vitro* for antifungal activity.

Methodology

Molecular cloning of *def* gene

In order to generate the *def* gene sequence, ESTs were used for this purpose. Twenty ESTs were collected from the *Triticum aestivum* cDNA library (http://www.ncbi.nlm.nih.gov). Based on the contig sequence generated by the assembly of these ESTs using CAP3 (Huang and Madan, 1999), a pair of primers flanking the *def* coding sequence (CDS) were designed by using Primer 3 (Rozen and Skaletsky, 2000), The pair of primers were designed as: *def*-forward primer, 5'-TCTCTGCTAGCAAGCGAGCAAGCAGAGAGAG-'3, included the restriction site for *Nhe*I and *def*-reverse primer, 5'-GTTATAAGCTT CATGGATGCTCTAGCCCAGT-'3, included *Hind*III restriction site (colored).

In order to amplify the cDNA of the *def* gene, the total RNA was extracted from the seeds of *T. aestivum* by using the TRI Reagent (Molecular Research Center, Inc., USA). Approximately 2 µg/mL of the total RNA were utilized in first-strand cDNA synthesis. The amplification of the *def* sequence was carried out by utilizing 5 µl 10x PCR reaction buffer; 1 µl dNTP (10 mM); 0.2µl Taq polymerase (5 U/µl); 1.5 µl MgCl2 (1.5 mM) (Invitrogen); 1 µl *def*-F forward and *def*-R reverse (10 µM) (Bio Basic). The cDNA (25 ng/mL) of liver library was used as a template. The PCR reaction was performed by using ependerouf–thermocycler. The PCR conditions were as follows: Pre-denaturation, 94°C for 2 min; denaturation, 94°C for 3 s; annealing, at 55°C for 30 s; extension 72°C for 1 min; 32 cycles; final extension at 72°C for 10 min. The PCR product was resolved on 1% agarose, and then the gel was purified by using QIAquick gel extraction kit (QIAGEN). The recombinant clones were sequenced by using the BigDye Terminator Cycle Sequencing Kit (Applied

Biosystems) on the ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Construction of the recombinant expression plasmid

The purified PCR product of *def* was cloned into the pGEM-T and transformed into *E. coli* DH5α competent cells. Recombinant clones were identified on LB agar plates containing ampicillin (50 µg/mL). The recombinant plasmid was extracted and purified from the *E. coli* DH5α cells by using the QIAprep Miniprep Kit (QIAGEN). PCR was performed to identify the positive colonies. Recombinant clones were confirmed by sequencing (Applied Biosystems). All the protocols are same to those recommended by the kit manufacturers. The double digestion was then performed for *def* with: 1 µl *Nhe*I (10 U/µl) and 1 µl *Hind*III (10 U/µl); 3 µl restriction enzyme buffer (10x), 0.3 µl BSA (0.1 mg/mL) (Promega), and 6 µl DNA template (pGEM-T vector + *def*) (700 ng/µl) to obtain a volume of 30 µl. The digestion mixture was then incubated at 37°C for 4 h, and then resolved on 1% agarose to confirm the released insert.

The pET-28b (+) plasmid (Novagen) was used for the cloning and expression of rdef in Rosetta- gami 2(DE3) expression host. The plasmid harboring *def* was digested with (*Nhe*I and *Hind*III). The digested *def* insert was subcloned into the pET-28b (+) (100 ng/µl) at a ratio of 1:3 utilizing T4 DNA ligase. The ligation mixture was incubated at 16°C for 16 h and transformed with 50 µl of *E. coli* DH5α competent cells. Recombinant clones were selected by plating them on LB agar plates containing kanamycin (30 µg/mL) (Sigma) for 16 h at the temperature of 37°C with shaking at 250 xg. The plasmids were extracted and purified from the overnight bacterial culture. The plasmid harboring the *def* gene was confirmed by double digestion with *Nhe*I, *Hind*III. Recombinant clones were confirmed by sequencing (Applied Biosystems) In all of sequencing steps, the protocols were essentially identical to those recommended by the kit manufacturers.

For small-scale expression, *E. coli* strains harboring the pET28b (+)-def plasmid were grown in (2 mL) of LB broth containing 30 µg/mL of kanamycin. The culture was grown overnight (16 h) at 37°C with shaking executed at 250 xg. The following morning, 10 mL fresh LB broth was inoculated with 500 µl overnight culture and incubated at 37°C for ~ 1 h, at which time the culture was in the mid-log phase (OD600 ~ 0.5). The rdef expression was induced by adding isopropyl-1-thio-b-D-galactopyranoside (IPTG) to a final concentration of 1 mM, and the mixture was left to grow for a further 4 h at 37°C. The cells were then harvested by the process of centrifugation at 6000 xg for 20 min. Later, the pellet was resuspended in 1 mL lysis buffer (50 mM Tris-base pH 8.0). The cell suspension was sonicated 6x for 10 s, each with a 20 s pause between sonication intervals, with a 6-mm diameter micro tip until the lysate became optically clear. The suspensions were centrifuged at 16000 xg, 4°C for 30 min. The supernatant (soluble protein fraction) and the pellet (insoluble protein fraction) were separated on 15% SDS-PAGE at 150V and stained with Coomassie brilliant blue R-250 (Sigma). For large-scale (1 L of LB broth + kanamycin 30 µg/mL) expression, 20 mL of an overnight def culture was added and grown at 37°C for ~ 1 h to reach the mid-log phase (OD600 ~ 0.5). IPTG was added to a final concentration of 1 mM and the incubation was continued for 4 h at 37°C.

Phylogeny of defensin

The defensin sequence of other plants was identified by using the generated

nucleotide sequence and PSI-BLAST (Altschul *et al.*, 1997) on the UniProt Knowledgebase (http://www.ebi.ac.uk/Tools/sss/psiblast/). The generated sequence was submitted to the NCBI for giving identification number. Next, a multiple sequence alignment (MSA) was created with the *T. aestivum* defensin sequence and other plant sequences by using T-coffee (Notredame *et al.*, 2000) (http://www.ch.embnet.org/software/TCoffee.html). The phylogenetic analyses were conducted by using PHYLIP (Felsenstein *et al.*, 1996) (http://evolution.genetics.washington.edu/phylip.html). The *Ginkgo biloba* was used as outgroups. The phylogenetic trees were constructed by using the neighbor-joining method (as implemented in NEIGHBOR) from distance matrixes calculated by the Jones-Taylor- Thornton method (using PROTDIST). The robustness of the trees was evaluated by bootstrap analysis of 1000 random iterations by using SEQBOOT, while MEGA4 (Tamura *et al*, 2007) (http://www.megasoftware.net/mega4/mega.html) was used to generate the consensus tree.

Expression and purification of recombinant def protein

Samples of (15 µl) from rdef protein were prepared under denature conditions in SDS sample buffer (10% SDS, 5% β-mercapitalethanol). The samples were boiled at 100°C for 5 min, and were then loaded and separated on 15% SDS-PAGE, with the electrophoresis which was then run for 1 h at 150V (Sambrook *et al*, 2001). Bacterial cells containing recombinant 6 · His-tagged def was resuspended in start buffer (20 mM potassium phosphate, pH 7.2, 0.5 M NaCl), sonified, centrifuged, and then loaded directly onto the Ni+- chelating affinity column pre-equilibrated with start buffer. After washing with five column volumes of start buffer, the 6 · His-tagged protein was eluted with stripping buffer (20 mM potassium phosphate, pH 7.2, 150 mM imidazole, 0.5 M NaCl). The recombinant defensin was examined, desalted and dried for later use.

Antifungal activity assay against *Puccinia striiformis*

Antifungal activity assays were carried out in Petri dishes containing about 20 ml of potato dextrose agar (Coda *et al.*, 2008). For the assay a piece of agar containing frontal mycelia of *Puccinia striiformis* was placed in the center of the plate. The plates were incubated for 6 h at 24°C. After this incubation period, sterile paper discs (3 mM, Whatman) were placed at a distance of 0.5 cm around the frontal mycelia. Different concentrations(50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml) of the protein sample were added to each disk. The plates were incubated at 24°C for (24h, 48h, 72h, and 96h) until mycelial growth had enveloped peripheral discs containing control buffer and had formed crescent of inhibition around the disc containing an effective concentration of antifungal agent. For the assay different concentration of the protein were spotted on discs. The extraction buffer (50 mM Tris, pH 7.4, 150 mM NaCl) was used as a control.

Results and Discussion

Sequence analysis of *Triticum aestivum* defensin

For sequence analysis, a PSI-BLAST search was performed by using the CDS of the *T. aestivum defensin* under the registered [GenBank: Q8L698_WHEAT] and a

total of 8 homologous defensin sequences were selected based on sequence length (full CDS over partial CDS or truncated protein), taxonomical group, and gene Table (1). These 8 sequences were aligned along with the *T. aestivum defensin* sequence, and the percentage of sequence identity to the was determined Table (1). The defensin protein sequences showed a relatively high degree of identity. The sole protein found in *Triticum durum* defensin (72%) and (65%) in species include (*Zea mays, Setaria italic, Saccharum officinarum*). These sequences have the highest identity to the *T. aestivum defensin* sequence followed by *Jatropha curcas, Ginkgo biloba,* (64%, 52%, respectively). The defensin sequence within the alignment having the lowest identity to *T. aestivum* is *Helianthus annuus* defensin sequence (51%) Table (1).

From the MSA, the maximum length of signal peptide region is 26 residues Figure (1) Table (2), which is varying between eight species studied. *Triticum. aestivum* and *T. durum* defensin sequences are 82 residues in length except sequences that have an five or four peptide deletion in species *Helianthus annuus* and *Jatropha curcas* and two peptide deletion in *Zea mays, Setaria italic, Saccharum officinarum* Table (1) .

In order to highlight the defined characteristics of the conserved amino acid residues among all members of the plant defensin, we performed the alignment of defensin proteins from several plants. Previous study (Gachomo *et al*, 2012) indicate that conserved residues are absolutely restricted to the eight cysteines at positions 36, 47, 53, 57, 67, 76, 78, and 82. A glycine residue(positions 26, 45, and 65) as well as a serine residues (38, 40, and 49) and an aromatic residue (F/H/Y) as depicted by black arrows. It support our study in which *T. aestivum* defensin sequence share same conserved regions except at positions 70 and 77 the aromatic residues (H and Y) conserved between *T. aestivum* and *Ginkgo biloba* Figure (1).These highly conserved residues likely to be essential for structural folding and/or stabilization of the proteins. Our results revealed that eight cysteine residues are strictly conserved among members of the selected plant defensin sequences and form strong disulfide bonds. Our characterization support previous study (Lopez *et al.*, 2003), which is regions between position 51 to 67, 68 to 78, and 79 to 82 represent the main loop, alfa-helix, and beta-sheet respectively.

Table (1): The defensin sequence homology

Sequence ID	Species name	Identity	Sequence length (aa)	E value
Q8L698_WHEAT	*Triticum aestivum*	-	82	-
C9E1C3_TRIDB	*Triticum durum*	72%	82	6e-26
F6MEN6_MAIZE	*Zea mays*	65%	80	1e-23
A2TH14_SETIT	*Setariaitalica*	65%	80	1e-23
B2CNV2_SACOF	*Saccharum officinarum*	65%	80	5e-23
D2D958_9ROSI	*Jatropha curcas*	64%	77	2e-17
Q670N7_GINBI	*Ginkgo biloba*	52%	80	9e-17
DFSD2_HELAN	*Helianthus annuus*	51%	78	3e-14

Table (2): Signal peptide cleavage position

FASTA-ID	Cleavage Position
Triticum aestivum	25
Helianthus annuus	24
Triticum durum	24
Zea mays	26
Setaria italica	26
Saccharum officinarum	26
Jatropha curcas	25
Ginkgo biloba	25

Phylogenetic analysis

The Neighbor Joining (NJ) phylogenetic analyses were performed using *Ginkgo biloba* (Q670N7_GINBI) as an outgroup. This sequence was chosen because it is the famous living fossils in the world and it has been widely used during the exploration of the origin of plant gene families (Shen *et al.*, 2004) Figure (2).

The phylogenetic analysis of *T. aestivum* defensin protein has never been performed. The phylogenetic topology revealed that the lineage leading from the outgroup branches into two main clades (Clade I and II). Clade I contains all the flowering plants (Monocots) and is supported with a bootstrap value of 75%, while the other clade (Clade II) contain Eudicots plants. Within (Clade I), two distinct separated groups appear. The first group refer to sub-family pooideae and it contain closely related species, which are *T. aestivum* (hexaploidy) and *T. durum* (tetraploidy). This group is supported with a bootstrap value of 87% with second group (sub-family panicoideae) containing *Zea mays, Setaria italica,* and *Saccharum officinarum* species. This topology indicate that *T. aestivum* defensin sequence share common features with the main ancestor *Ginkgo biloba* and defensins in group panicoideae arise after the divergent from pooideae Figure (2).

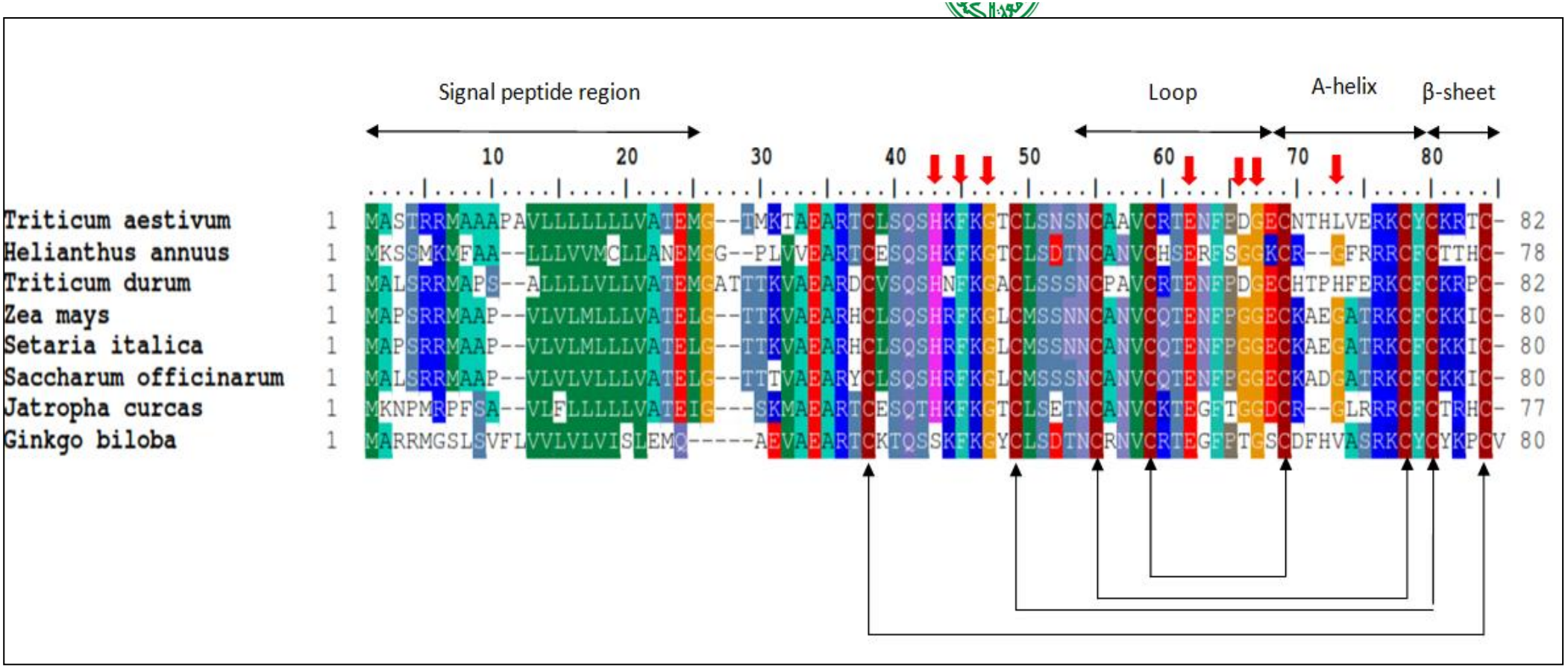


Figure (1): Sequence comparison between plant defensin protein superfamily. Identical amino acids are shadowed. The invariable cysteine residues are depicted in dark brown background. The disulfide bond connectivity is shown below by connecting lines. Seven most conserved positions and essential for structural folding and/or stabilization of the proteins are highlighted with red arrows on the top of the alignment.

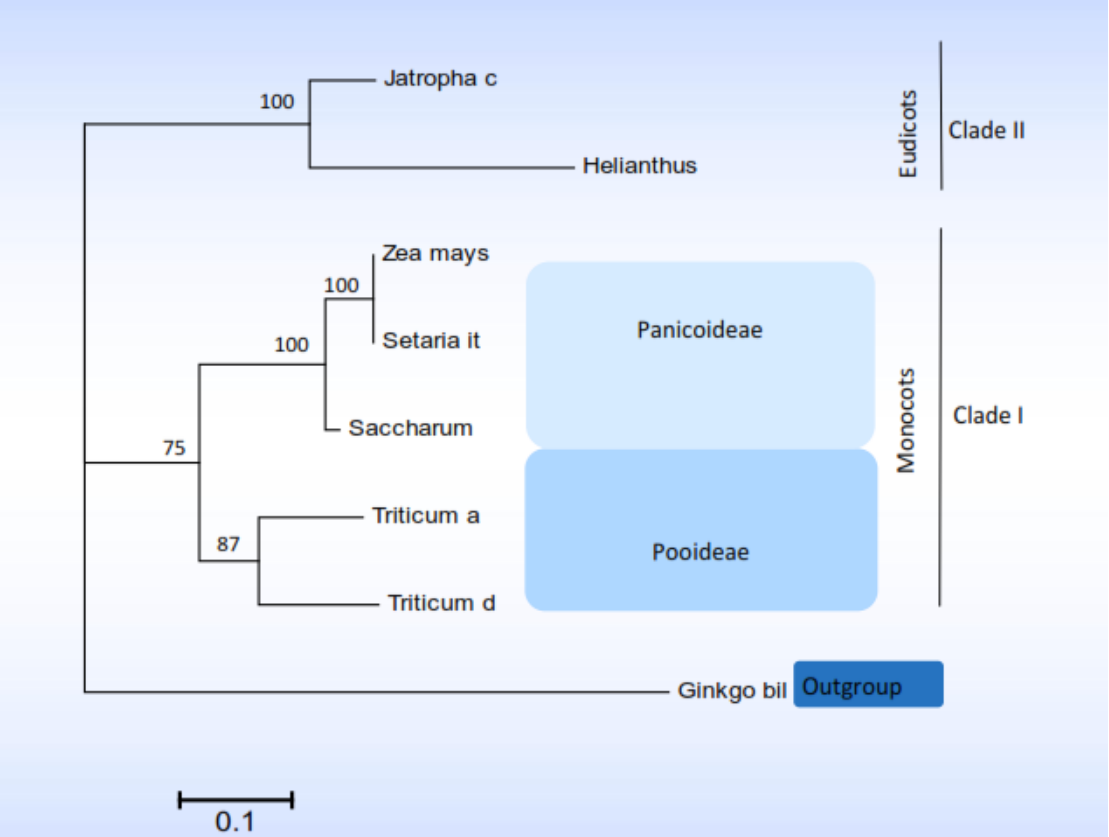


Figure (2): Neighbor-joining phylogenetic tree.

The phylogenetic tree was constructed from the aligned *Triticum aestivum defensin* sequence with other plant defensin protein sequences using the neighbor-joining method with distance calculated using the Jones-Taylor-Thornton model. Bootstrap values are shown above the respective nodes based on a percentage of occurrences in 1000 replications. The red circle refer to the target gene protein sequence.

CDS cloning and in vitro expression of rdef protein

The results showed that the amplified open reading frame (ORF) of the *T. aestivum defensin* is 249 bp in length Figure (3). It encodes a protein of 82 amino acids. The target gene was cloned into pET-28b vector for protein expression. The recombinant protein was then over-expressed by IPTG induction. The Defensin protein obtained from over-expression in *E. coli* carried an N-terminal extention containing a 6- His tag, which was used to simplify purification via affinity chromatography. Results of recombinant expressed and purified protein was analysed with SDS-PAGE gel electrophoresis showed that there was only one protein band with the apparent molecular weight about ~13 kDa Figure (4). The absorbance ratio of the native def fraction (A650/A280) purified with hydroxylapatite chromatography was 4.1, which could be considered as pure defensin.

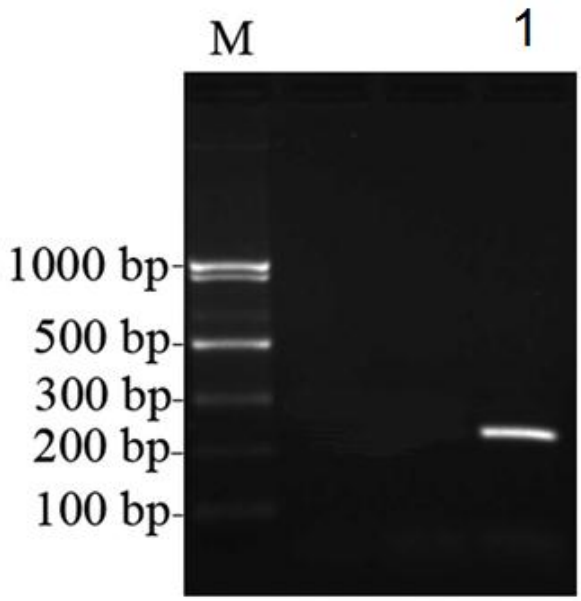


Figure (3): Amplification and double digestion of def gene. Lane 1: Amplified def with a size of 249 bp. M: DNA ladder (100-1000bp)

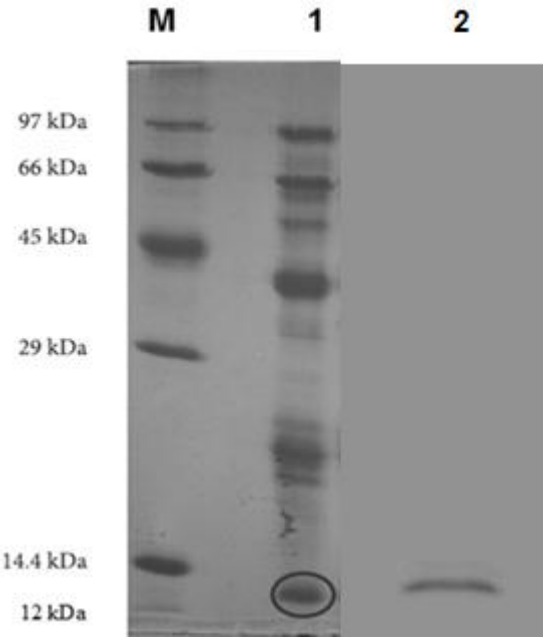
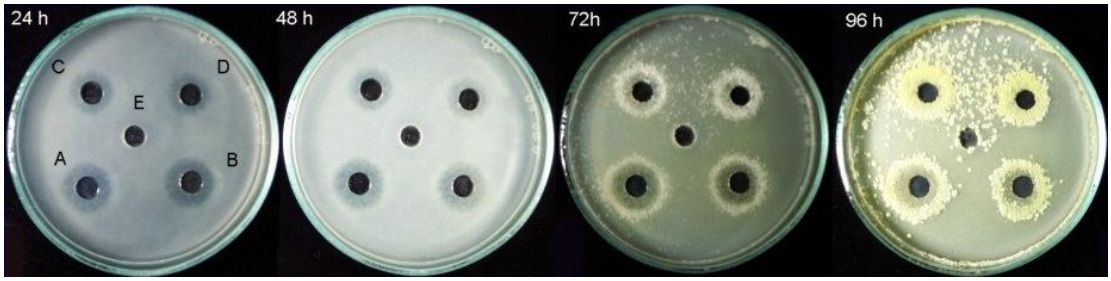


Figure (4): Expression and purification of def protein. Lane 1: def protein with a size of ~13-kDa was expressed form E. coli Rosetta-gami 2(DE3) and resolved on 15% SDS-PAGE. Lane 2: The purified rdef protein that eluted from strong anion exchange column (Q Sepharose). M: Protein marker (12-97 kDa).

Antifungal activity against *Puccinia striiformis*

The possible toxicity of the purified recombinant protein, rdef was tested using the fungus, *Puccinia striiformis*. It is apparent from the Figure (5) that the recombinant protein inhibited the mycelial spread of *Puccinia striiformis* and formed a crescent of growth inhibition, whereas the control (disc E) had no
detectable effect on the fungus. It was clearly shows that 50 µg/ml concentration of rdef inhibits the mycelial spread of *Puccinia striiformis* and shows a crescent of growth inhibition around the disc A. As the concentration decreases, there was a decline in the inhibition range as observed in disc C (12.5 µg/ml) and disc D (6.25 µg/ml). our results confirmed that antifungal activity is belong to rdef only compared to control.

Figure (5): Effect of rdef on hyphal extension on the growth of *Puccinia striiformis* on complete medium (CM: 0,2% peptone, 0,1% yeast extract, 0,1% NZ-amine A, 2% glucose, 0,05% KCl, 0,04% MgSO4 .7H2O, 0,15% KH2PO4, pH=6.5) after incubation of 24, 48, 72, 96 h, at 25°C. The paper discs have the following concentrations: 50 µg/ml (**A**), 25 µg/ml (**B**), 12.5 µg/ml (**C**) and 6.25 µg/ml (**D**). 100 µl CM broth without rdef was used as control (**E**).



The exact mechanisms underlying the antifungal activity exerted by plant defensins are not known, but there is evidence that plant defensins bind to a specific receptor in the fungal membrane. Plant defensins could interact selectively with phosphorylinositol containing sphingolipids or glycosylceramides and then trigger the death of fungal cells through binding to the target cell membrane (Heung *et al.*, 2006). Thus, the direct antifungal effect of defensins in vivo is likely to occur mainly in the phagocytic Vacuoles of phagocytes and on the Surface of skin and mucosal epithelia , where there is low ionic strength .

Other previous studies identified that all defensin tend to have the capacity to kill or inactivate a particular spectrum of bacteria, fungi or some enveloped viruses *in vitro* and are generally considered to be direct effectors of innate antimicrobial immunity (Jenssen *et al.*, 2006) with the exception of β – defensins have reduced antimicrobial activities in the presence of physiological concentration of salt (i . e. 150 mM of Nacl) (Jung *et al.*, 2011) thus, the direct antimicrobial effect of defensins *in vivo* is likely to occur mainly in the phagocytic Vacuoles of phagocytes and on the Surface of skin and mucosal epithelia , where there is low ionic strength .

Early studies on defensins (Patterson *et al.*, 1981), (Lehrer *et al.*, 1989) revealed the general mode of action as being permeabilization of the plasma membrane and consequent leakage of cell content. It have been demonstrated that defensine A – Perturbs

the cytoplasmic membrane of *M . luteus* to Cause loss of Cytoplasmic K + , membrane depolarization, a decrease in cytoplasmic ATP, and inhibition of respiration, permeabilization of energized M . luteus was reported to cause the transmembrane protein to drop from – 200 mV to – 110 mV (Potential inside the cell with respect to that outside the cell) (Li *et al.*, 2013).

Conclusion

Our *in silico* analysis revealed that *Triticum aestivum* defensin is share a very high degree of homology with other plants studied. The most important defensin gene region was fully conserved including eight cysteine residues. This support the main function and similarity to other plants. Meanwhile, the phylogenetic analysis was strongly support this results. The recombinant defensin protein showed a strong antifungal activity against *Puccinia striiformis*, indicates that this protein is vital with promising agro-biotechnological and pharmaceutical applications.

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