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# Functional Genomics for Orobanche Tolerance in Faba Bean (*Vicia faba* L.)

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## ABSTRACT

Two faba bean cultivars were studied for orobanche (*Orobanche crenata*) tolerance. A greenhouse experiment was conducted using two Egyptian faba bean (*V. faba* L.) cultivars infected by *Orobanche crenata* in pots. The results of the control plants (without orobanche infection) and infected plants (with or without orobanche attachment) showed that the susceptible cultivar is Giza 3 and the tolerant one is Giza 843 according to the number of orobanche tubercles per plant. Differential display (DD)-PCR was utilized in order to isolate mRNAs of stress-related gene fragments. The results showed up-regulated and down-regulated expressions for DD fragments in both cultivars Giza 3 and Giza 843 under control and orobanche treatments (with and without attachment). Some gene-related fragments were identified and aligned with BLAST data-base. They were similar to some known genes or proteins related to different-stresses tolerance in different genera. They may play a major role in orobanche infection tolerance mechanism and can be used for isolation of orobanche-tolerance related genes (full length) by using isolated cDNAs as probes in the future. Finally, Fragment 6 (383 bp) from G.3 was aligned on EST database and a BLAST-submission was obtained.

**Key Words:** Faba bean, *vicia faba, orobanche crenata,* differential display, submission.

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## INTRODUCTION

Since the dawn of civilization, legumes together with cereals have been fundamental to the development of modern agriculture providing protein in human diet, edible oils and fodder and forage for animals. Faba bean (*Vicia faba* L.) is one of the oldest legume crops mainly grown for human and animal dietary needs. Like other grain legumes, faba bean contributes to sustainable agriculture by fixing atmospheric nitrogen in symbiosis with soil bacteria. This unique ability reduces the dependence of farmers on extensive use of chemical fertilizers protecting soil and water quality. In addition, legumes play a critical role in crop rotation, improving soil physical conditions and decreasing the amount of diseases and weed populations which in turn leads to lower consumption of herbicides and fungicides. Large-scale production of faba bean has been historically hindered by low and unstable yields as well as by susceptibility to several diseases *(Duc, 1997, and Knott, 1997).*

The genus *Vicia* comprises a number of species ranging from 180 to 210 that are distributed throughout the temperate zone *(Hanelt and Mattin, 1989).* At the level of morphological variation, genus *Vicia* is divided into several sections *(Hanelt, 1983).* Biotic stresses cause severe problems for faba bean production. The parasitic weed broomrape (*Orobanche crenata*) is a yield-limiting factor in some fields, in which could wipe out the crop *(Saber et al., 1998).* The infested area in Behera governorate, for instance, was estimated to be 65.5% with total yield loss of about 19,000 tons *(Zaitoun et al., 1991).* Broomrapes (*Orobanche* spp.) are parasitic weeds that have a devastating effect on many important crops *(Cubero, 1983).* It is holo-parasites, completely lacking chlorophyll and causing significant reductions in yield and quality. The seedlings attach to the root system of the host plant, establish a connection with the vascular system of the host via a specialized organ (haustorium) and deprive the host of water, mineral nutrients and metabolites *(Parker and Riches, 1993).* Differential display (DD) is one of the methods for analyzing gene expression in eukaryotic cells and tissues. DD has been widely applied to study changes in mRNA expression induced by temporal developments, biotic and abiotic factors *(Liang and Pardee, 1992, Liang*

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*et al., 1992, Guimaraes et al., 1995, McCarthy et al., 1995, Hu et al., 1996 and Liu and Baird, 2003).* This powerful technique simultaneously screens for both up-regulated and down-regulated transcripts in multiple cell populations under different developmental and environmental conditions.

This study aims to identify two faba bean cultivars as tolerant and susceptible to Orobanche infection, obtain stress-related cDNA fragments using differential display technique, isolate these Expressed Sequence Tags (ESTs) fragments, clone and define the sequence of isolated ESTs fragments and determine the gene functions for each EST using bioinformatics approaches.

## PATIENTS AND METHODS

**Plant Materials:**

Two faba bean (*Vicia faba* L.) cultivars; Giza 843 (G.843) and Giza 3 (G.3) were supplied by Leguminous Crops Research Department (LCRD), Field Crops Research Institute Agricultural Research Center (ARC), Giza, Egypt. *Orobanche crenata* seeds were supplied by Grass Research Institute, Agricultural Research Center (ARC).

**Pot Experiments:**

The effect of infection by *O. crenata* was studied in pot experiments in a greenhouse maintained at 20±5°C. Faba bean plants were grown individually (one per pot) using peat: perlite: vermiculite (2 :2 :1, w : w) or sterile soil in the oven at 60°C mixed with 400 mg (about 90,000 seeds) of *O. crenata* seeds collected during the previous season from plants parasitizing faba bean *(Rubiales et al., 2004 and 2006).* Each cultivar was represented by 120 pots (40 controls, 40 with attachment and 40 without attachment) in a completely randomized design. Three months after sowing, the plants were removed from the pots, the roots gently washed with water and the number of *O. crenata* tubercles per plant was counted.

**RNA isolation and mRNA Differential Display:**

RNA extraction was made using the RNAeasy RNA extraction kit. Roots of the two faba bean cultivars were harvested from control as well as under parasite (*O. crenata*) treatments (with and without attachments). Total RNA was isolated from frozen tissues using an RNeasy kit (Qiagen, USA). DNA contamination was removed using DNase free RNase (DNase I). mRNA differential display *(Liang and Pardee, 1992)* was conducted using RNA image kit (GenHunter Corp.) according to the manufacture>s instruction, except that the anchor primer (T11G,) was fluorescence-labeled (Metabion Corp). DD-PCR amplification for each primer pair was performed twice from RNA samples isolated separately at each time point. PCR products were separated on 6% denaturing polyacrylamide gels and visualized using Green leaser beam (532 nm) for Fluorescence excitation using Typhoon scanner (Amersham, USA). Location of bands of interest that shows differential expression patterns were determined using gradient transparent sheets.

**Cloning:**

Differentially displayed cDNA bands were excised from polyacrylamide gels with a scalpel and eluted in 100µl sterilized water for 1 hour at room temperature, followed by 15 min at 94°C. cDNAs were recovered by ethanol precipitation, re-dissolved in 20µl nuclease-free water and 2µl aliquot was used for re-amplification. Two µl of successful PCR reaction were sub-cloned into pGEM-T easy vector system (Promega, USA) according to the manufacturer manual.

**Sequencing and Sequence Alignments:**

The cloned cDNA fragments were sequenced using M13 primers with the ABI PRISM Big Dye Terminator (PE Applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). All inserts were sequenced on both strands and DNA sequences were compared with sequence databases using BLAST-N and BLAST-X algorithms *(Altschul et al., 1997).*

## RESULTS

**Performance of the Two Faba Bean Cultivars under Orobanche Infection:**

A greenhouse experiment was conducted using the two Egyptian faba bean cultivars under infection of *Orobanche crenata* comparing with control (without infection) in pots. Infected plants were taken from the pots to examine the presence of tubercles (with tubercles) or absence (without tubercles). Figures (1, 2 and 3) exhibited the differences between faba control plant (without infection) and the infected plants (with or without orobanche attachment) for the two cultivars; G.3 and G.843. The results indicated that cultivar G.3 was highly infected and gave many tubercles in the infected pots (Figures 1 and 2) compared with the control (without infection), while G.843 showed lowest infection (Figure 3). These results confirmed that G.843 can be considered as orobanche tolerant cultivar, while G.3 was very susceptible cultivar.

**Differential Display:**

Faba bean genes whose expression was regulated by infected Orobanche stress were studied by differential mRNA display. The fluorescence-labeled anchor primer (T11G) was used to target the polyA tail at the 3` end of stress-related genes and

the random primer (ARP1), attempting to hit the open reading frames (ORFs) at the 5` end of these genes, was used. Figure (4) showed DD-PCR results visualized using Green leaser beam (532 nm) for fluorescence excitation using Typhoon high-quality confocal optical imaging system scanner.

**Expression patterns of detected DD-cDNAs of susceptible cultivar (G.3):**

A total of approximately 100 bands were observed along the one DD-gel and as high as 11 of them (11%) showed differential amplification between treatments. Figures (4 and 5) indicate a closer view of the 11 DD bands. Many other DD fragments were shown but expression patterns of the duplicated samples were inconsistent and thus excluded from further analysis. DD fragments were classified into three patterns (groups) of expression as shown in (Table1). These groups indicated varying up-regulated and down-regulated patterns of stress-related (induced and repressed) genes at different types of treatments (control, without and with orobanche attachment). One out of the three expression patterns indicated down regulation of two stress-related gene fragments (1 and 2) at without and with attachment compared with normal expression control. The second expression pattern showed up-regulated DD of five stress-related gene fragments (3 - 6 and 11) at without orobanche attachment, while exhibited down-regulated pattern at with treatment compared with none expression control. The third expression pattern showed up-regulated DD of four stress-related gene fragments (7 -9 and 10) at without and with orobanche attachments compared with none expression control.

***Expression patterns of detected DD-cDNAs of tolerant cultivar (G.843):***

A total of approximately 70 bands (Figure 4) were observed along the one DD-gel and as high as six of them (11.66%) showed differential amplification between treatments. Figures 4 and 6 indicate a closer view of the six DD bands. Many other DD fragments were shown but expression patterns of the duplicated samples were inconsistent and thus excluded from further analysis. DD fragments were classified into four patterns (groups) of expression as shown in Table 2. These groups indicated varying up-regulated and down-regulated patterns of stress-related (induced and repressed) genes at the three types of infections (control, without attachment and with attachment) to the biotic stress of *Orobanche crenata*. Group 1 included two cDNA fragments (12 and 13) with normal expression at control, while showed down-regulated DD at without orobanche attachment and with attachment exhibited up-regulated DD ones. Group 2 involved one cDNA fragment (17) with normal expression at control, while showed none expression for both without and with attachments. Group 3 contained one cDNA fragment (16) with none expression at control, while gave up-regulated expression at without attachment and down-regulated expression at with attachment. Group 4 included two cDNA fragments (14 and 15) with up-regulation at without and with attachments compared with none expression for their corresponding genes at control.

**Cloning and sequence homologies of the differential display cDNA clones:**

Four cDNA fragments out of the previous ones were cloned into pGEM-T Easy vector and sequenced with M13 primers. DNA sequencing reactions were conducted for these fragments which were over 100 bp (3, 6 from cultivar G.3 and 13 and 14 from cultivar G.843) as shown in (Figure 4. Table 3). summarizes the homology search results based on computer analysis using BLAST site from National Center for Biotechnology Information (NCBI). Scanning of fragment 3 (control of susceptible cultivar G.3) at the GeneBank data showed significant homology with *Medicago truncatula* cDNA clone pGESD4D18 5> end, mRNA sequence, with score = 120 bits, E-value= 2e-24 and results of alignment and Identities were 75/81 (92%). Scanning of fragment 6 (with attachment of susceptible cultivar G.3) showed significant homology with *Oryza sativa* subsp. indica root seedling cDNA clone Osic22 3>, mRNA sequence, with score= 603 bits, E-value = 3e-169 and the results of alignment and Identities were 362/379 (95%). Scanning of fragment 13 (control of tolerant cultivar G.843) showed significant homology with *Medicago truncatula* clone mth2-14b-12, complete sequence with score= 69.8 bits, E-value= 6e-09 and the results of alignment and Identities were 79/106 (74%). At the same time, it showed homology with *Vicia pannonica transposon* Ty3/gypsy-like LTR retrotransposon sequence, with score= 68.0 bits, E-value= 2e-08 and the results of alignment and Identities were 82/112 (73%). Fragment 13, also, in the GeneBank (protein sequence database) showed significant homology with Peptidase aspartic, active site *(Medicago truncatula)* with score= 68.2 bits, E-value= 2e-10 and the results of alignment and Identities were 38/70 (54%).

**Submission of fragment 6:**

Fragment 6 (383 bp) was aligned on EST database and a submission was obtained (accession number GE 469665). It is considered the first fragment which isolated from cultivar G.3 (susceptible) under orobanche infection and is cited now on BLAST-DataBase.

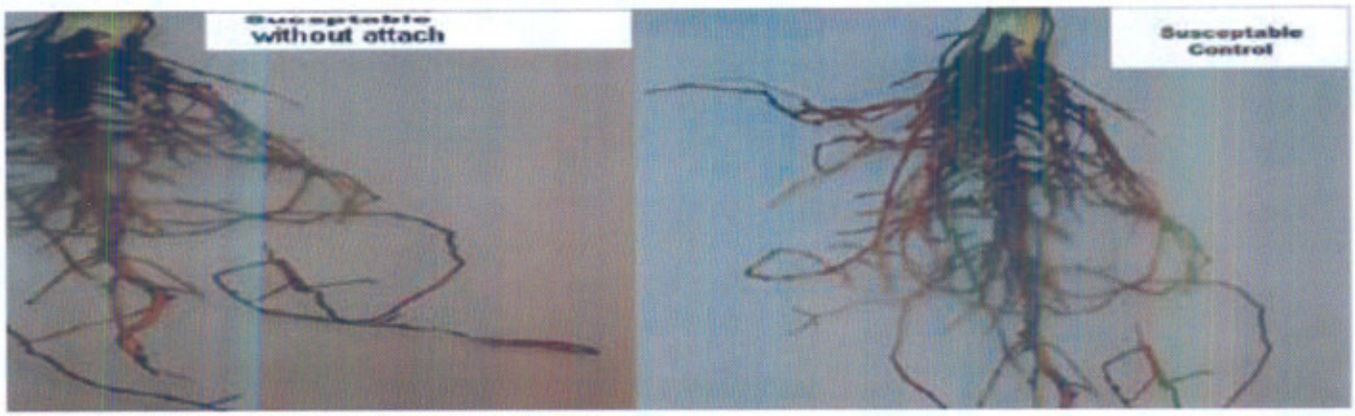



**Figure 1:** Giza 3; control and without Orobanche attachment.

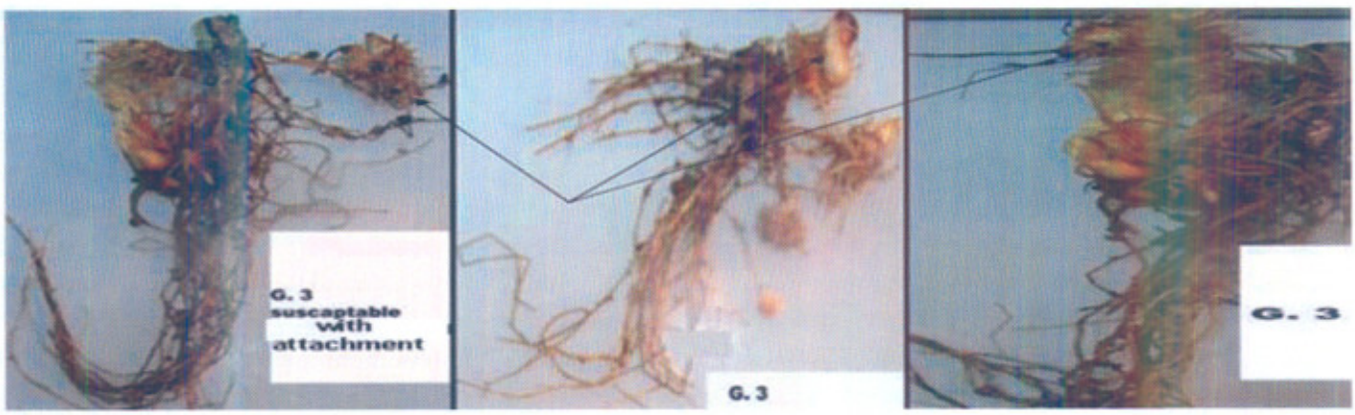



**Figure 2:** Giza 3 with Orobanche attachment.

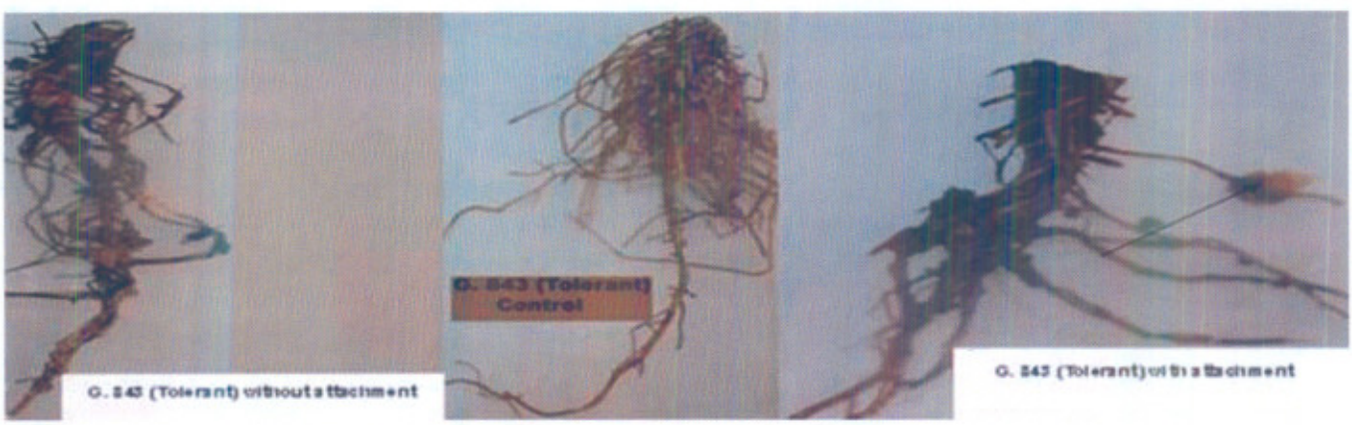
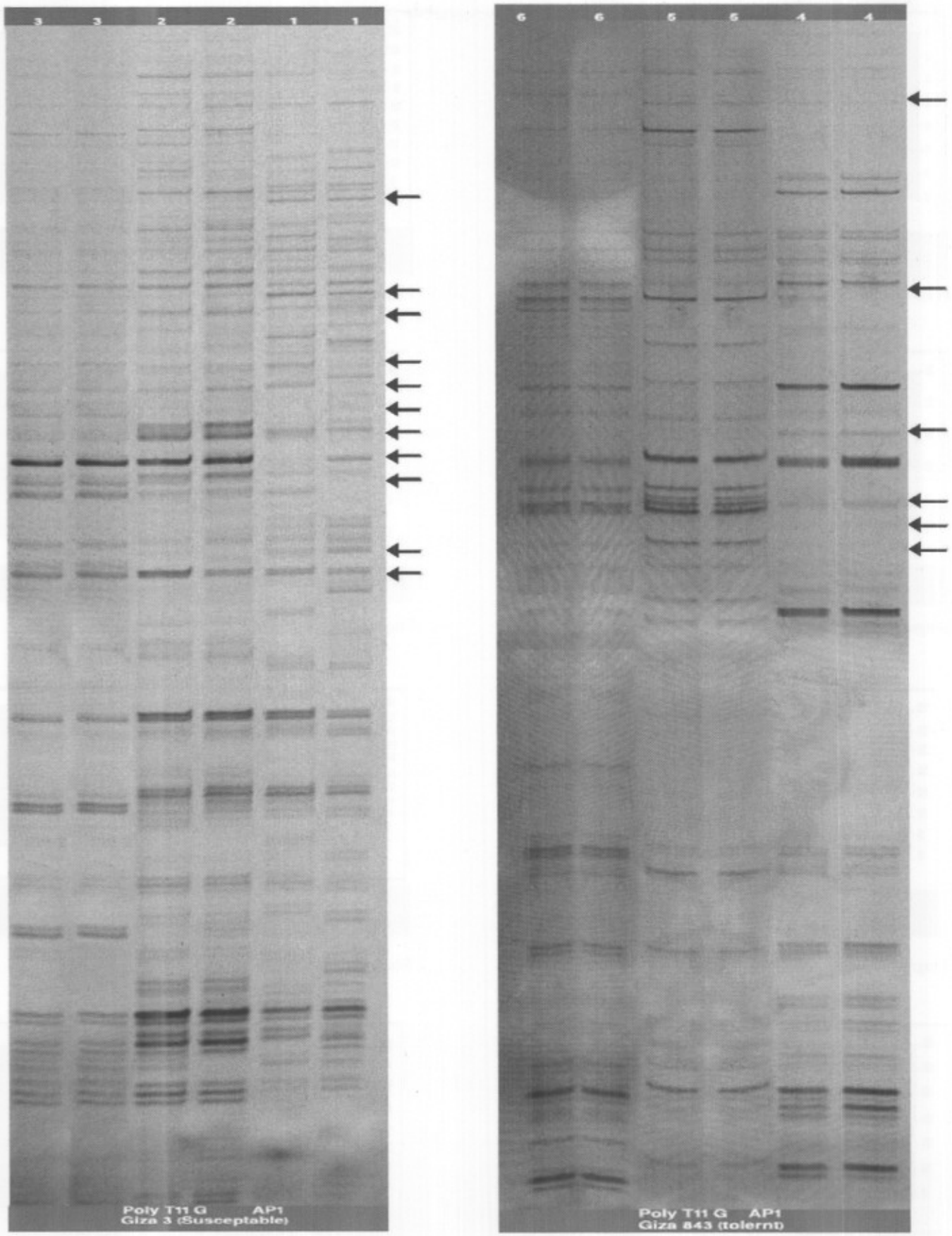



**Figure 3:** Giza 843 control, without and with Orobanche attachment.

**Table 1:** Expression patterns of the obtained DD fragments from Giza 3.

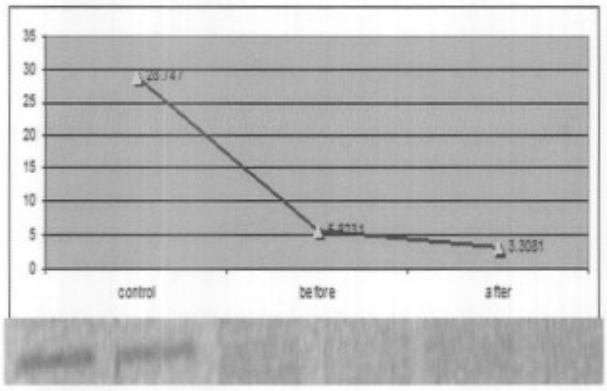
| Pattern | Control | Without attachment | With attachment | DD fragments |
|---|---|---|---|---|
| 1 | normal | down-regulated | down-regulated | 1,2 |
| 2 | none | up-regulated | down-regulated | 3, 4, 5, 6 and 11 |
| 3 | none | up-regulated | up-regulated | 7, 8, 9 and 10 |



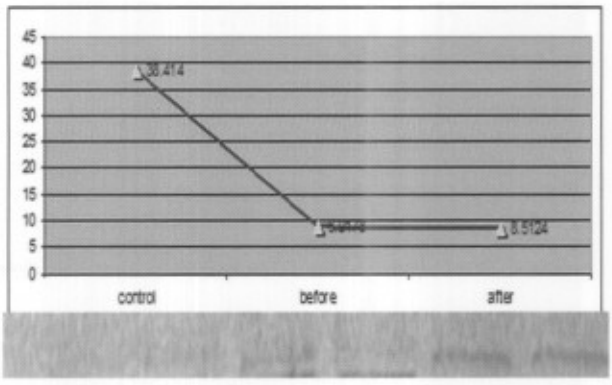

**G.3:** 1- control 2- without attachment 3- with attachment, each sample was replicated twice.

**G.843;** 4- control 5- without attachment 6- with attachment, each sample was replicated twice.

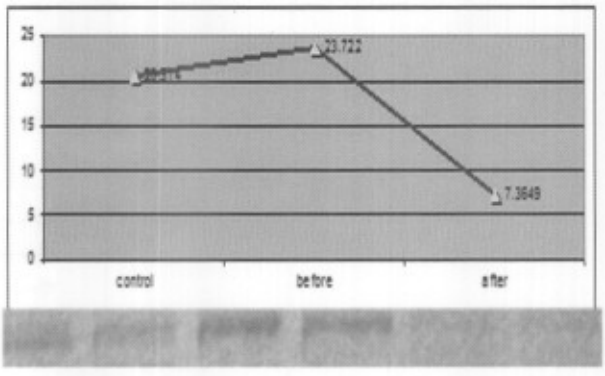
**Figure 4:** DD-polyacrylamide gels of root cDNAs under control and orobanche stress (without attachment and with attachment) in cultivars G.843 (Tolerant) and G.3 (Suceptible) conditions used primer T11G and AP1 arrows indicate a number of differentially expressed bands on a duplicate basis.




Fragment 1.




Fragment 2.

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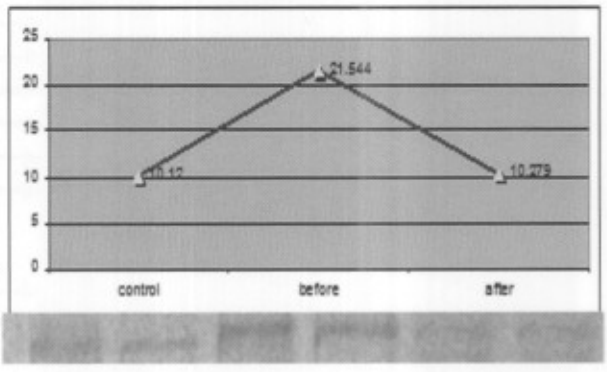
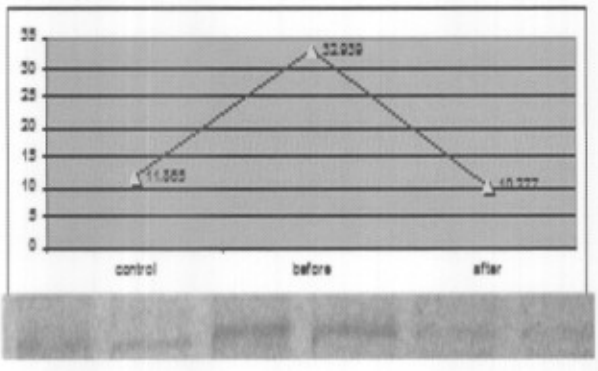



Fragment 4.

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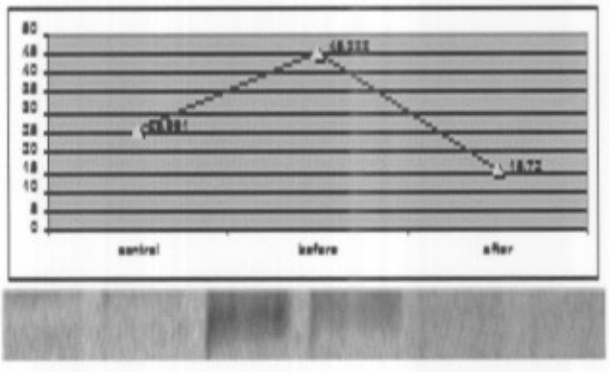


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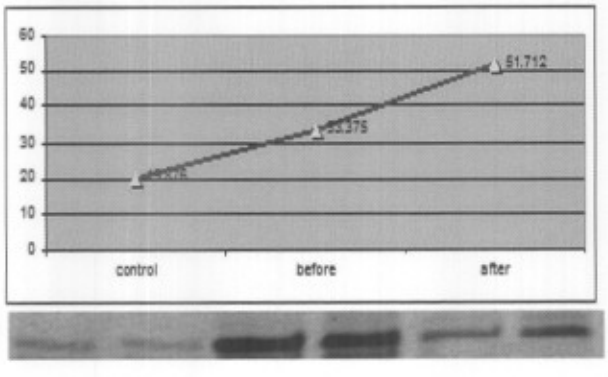


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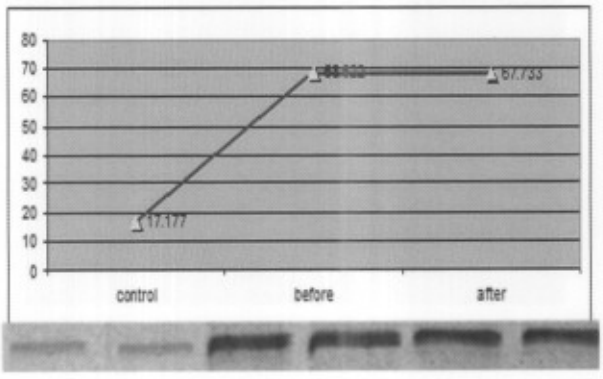


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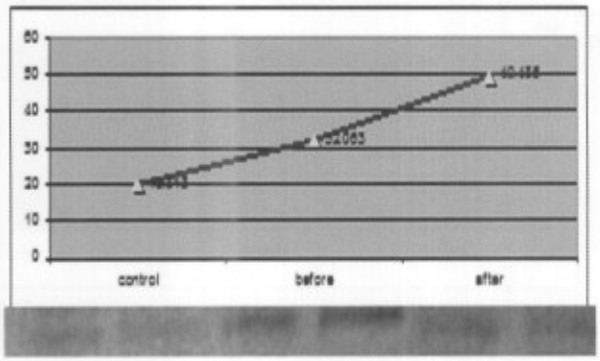


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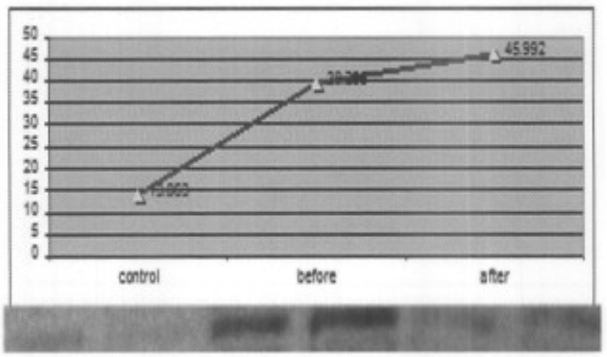


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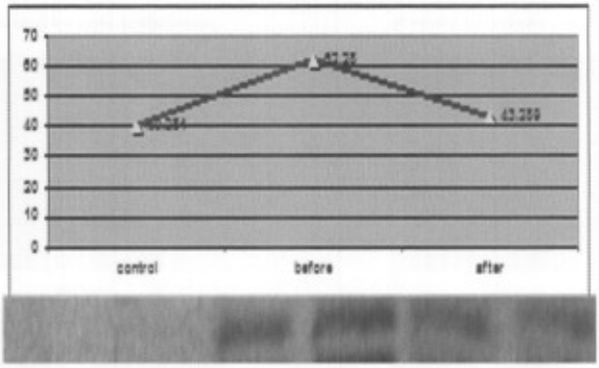



Fragment 11.

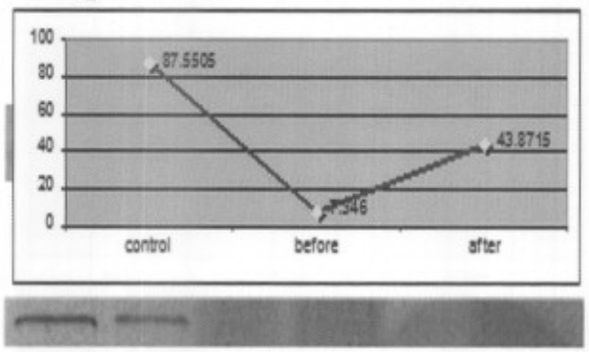
**Figure 5:** The 11 DD fragments and their expression profiles under control and without and with attachments of cultivar Giza 3.

**Table 2:** Expression patterns of the obtained DD fragments from Giza 843.

| Pattern | Control | Without attachment | With attachment | DD fragments |
|---|---|---|---|---|
| 1 | normal | down-regulated | up-regulated | 12 and 13 |
| 2 | normal | none | none | 17 |
| 3 | none | up-regulated | down-regulated | 16 |
| 4 | none | up-regulated | up-regulated | 14 and 15 |

**Table 3:** The four DD-fragments and their homology search results.

| Frag. no. | Molecular size (bp) | Homology search results (GeneBank accession number; E-value) | Function |
|---|---|---|---|
| 3 | 136 | Medicago truncatula cDNA clone pGESD4D18 5' end, mRNA sequence, 2e-24 | Unknown protein |
| 6 | 383 | Oryza sativa Indica Group cDNA clone Osic22 3', mRNA sequence, E-value = 3e-169 | Unknown protein |
| 13 | 253 | Medicago truncatula clone mth2-14b12, complete sequence, E-value = 6e-09 | Peptidase aspartic, active site |
| 14 | 207 | Vicia pannonica transposon Ty3/gypsy-like LTR retrotransposon sequence, E-value = 1e-34 | gag/pol polyprotein |

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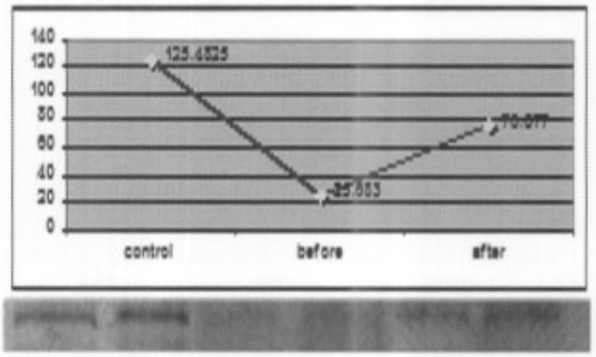
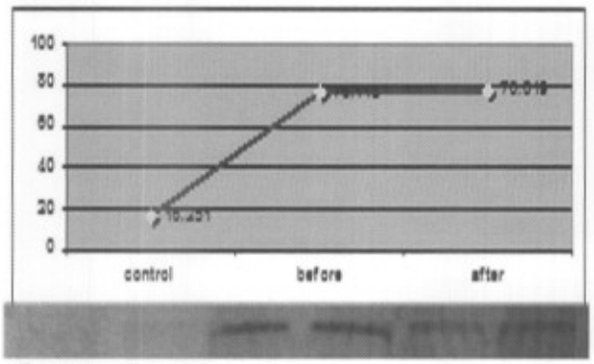
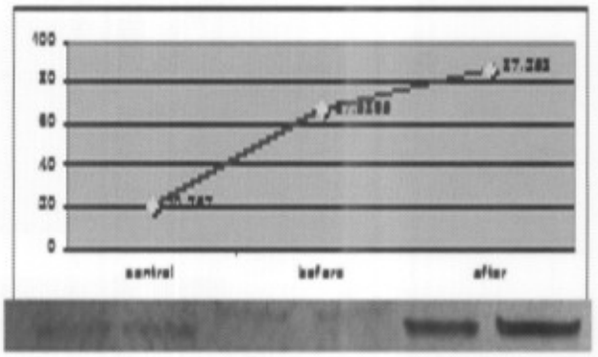


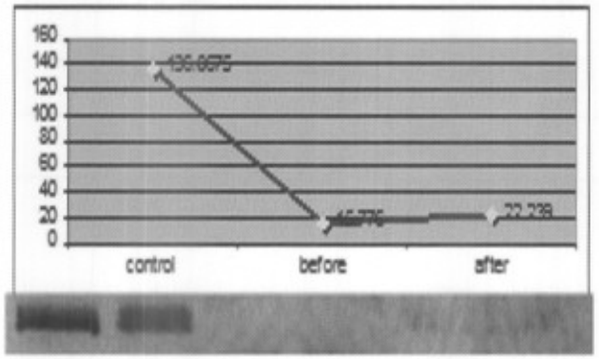

Fragment 13.



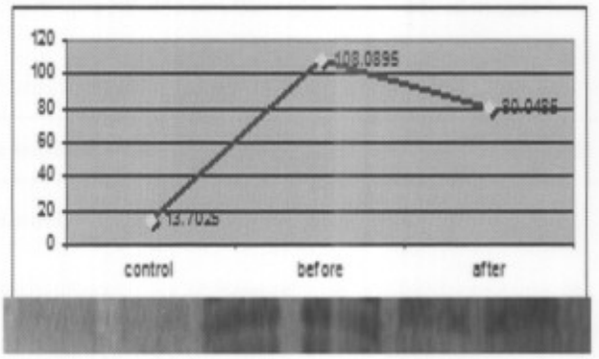

Fragment 15.




Fragment 14.




Fragment 17.




Fragment 16.

**Figure 6:** The six DD fragments and their expression profiles under control and without and with attachments of cultivar Giza 843.

## DISCUSSION

Comparable findings were obtained with Boorsma *(1980), Abdalla (1982), Attia (1992), Borg et al. (1994), Saber et al. (1998), Khalil et al. (1994), Abdalla and Darwish (1996 and 1998) and Ghalwash (2003).* They reported that the number of *Orobanche spikes*/plant increased in the susceptible cultivars and vice versa. In addition, *Gillanders et al. (2002)* stated that stress sensitivity or resistance depends on the species, the genotype and the developmental stage of the plant. *Kawasaki et al. (2001)*, who isolated, cloned, characterized and gave Gene expression profiles during the initial phase of salt stress in rice. Among these stress-responsive genes, 40–50% of them code for unknown proteins, e.g., Rab-related proteins and dehydrins. The identification of these unknown genes has not only led to the discovery of novel gene function, but also provides new information for a better understanding of the mechanism(s) involved in plant environmental stress responses. The roles of these proteins in response to environmental stimuli will be better understood as more of these genes, their expression and interaction are known *(Liu and Baird, 2004)*. Comparable results were reported by *Shokry et al. (2007)*, who isolated of some Expressed Sequence Tags (ESTs) related to salt tolerance in rice, cloned and sequenced of isolated ESTs and Determined gene functions for each EST using bioinformatics approaches, *Eissa et al. (2007)*, who used differential display technique and reported the same results and *Edris et al. (2009)*, who isolated of some expressed sequence tags (ESTs) related to salt tolerance in yeast, cloned and sequenced of isolated

ESTs and Determined gene functions for each EST using bioinformatics approaches by differential display technique.

*Koyano and Nakano (2008)* found that peptidase aspartic, is an inhibitor of the active sites of aspartic acid. Scanning of fragment 14 (with attachment of tolerant cultivar G.843) showed significant homology with M13-seq salt-treated full-length cDNA library of wild soybean Glycine soja cDNA, mRNA sequence with score = 104 bits, E-value= 3e-19 and the results of alignment and Identities were 125/168 (74%), Fragment 14 also, in the GeneBank (protein sequence database), showed significant homology with gag/ pol polyprotein with score= 89.7 bits, E-value= 6e-17 and the results of alignment and Identities were 56/67 (83%). *Anderson and Lever (2006)* confirmed that these proteins control the translation process. Finally, as noted above, some cDNA fragments which play a role in orobanche stress tolerance and resemble some genes or proteins on BLAST-database were isolated by using florescent differential display technique. They may play a major role in orobanche infected tolerance mechanism and can be used for isolation of orobanche-tolerance related genes (full length) by using isolated cDNAs as probes in the future.

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