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EXPRESSION OF DEFENSIN GENES IN EGYPTIAN WHEAT *(Triticum aestivum)* VARIETIES DURING GRAIN GERMINATION.

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ABSTRACT

The Seven commercial Egyptian wheat varieties : Misr1, Giza168, Sakha94, Sids1, Gemmiza7, Gemmiza11, and Shandawel1) were used to investigate the expression of defensin genes during seed germination. Three specific pairs of primers designed to amplify fragments of their respective defensin genes (PDF3, PDF5, and PDF30)were used. The reverse primers were utilized to synthesize cDNA from total RNA isolated from wheat shoots. PCR was used with cDNA to amplify fragments of defensins genes from the seven varieties. Results of this study showed that there was difference in defensin gene expression among the seven varieties. This included absence of PDF5 expression in Sids1and absence of PDF30 expression in Gemmiza7. Also, variations in the gene expression level among species were detected. Misr1 showed low expression, whereas Shandawel1 gave the highest expression of the three studied genes. Other varieties represented various degrees of expression for the three genes. It seemed that the difference in defensin gene expression would be related to the resistance of wheat varieties to diseases and a biotic stresses. This study would contribute information about defeinsin gene expression as a biodiversity tool in wheat breeding programs and variety evaluation.

INTRODUCTION

Wheat *(Triticum aestivum)* is the most important food crop in the world and is considered the first strategic winter crop (www.FOA.org). Many factors determine the production and quality of wheat including biotic and abiotic factors. Biotic factors mainly include wheat varieties and wheat diseases. Defensins are short peptides that represent subgroup of antimicrobial peptides widely expressed in eukaryotic cells for defense against bacteria and fungi (Yamaguchi and Ouchi 2012). Many defensins have been studied from various sources according to the defensins knowledge database (http://defensins.bii.a-star.edu.sg). Now , there are 564 characterized defensisns distributed as; 273 from vertebrates, 181 from in vertebrates, 61 from plants, 6 defensins-likes, and 43 unclassified. The 61 characterized defensins from plants show that these molecules are understudied in the rich plant sources worldwide.

The size of plant defensins ranges from 45 to 54 aa and have very diverse amino acid sequence with conserved tertiary structure with α-helix and three antiparallel β-sheets (Valente *et al*. 2013; De Beer and Vivier, 2011). The tertiary structure of defensins has a structure known as the cysteine rich motif which is conserved in all plant defensins and other eukaryotes. Plant defensins represent extreme primary diversity sequence, however they have some common structural features including, lacking of a high amino acid signature that relate to their diverse activities, defensins have been co-evolved through a process that accommodate mutations to avoid

pathogen resistance, They are amphipathic positively charged at physiological pH that facilitates their interactions with the anionic head groups of microbial membrane lipids (Valente *et al*. 2013).

Defensins have been isolated from various plant organs including roots, flowers, and fruits (De Beer and Vivier 2011; De Beer and Vivier 2008). They play an important role in the protection of germinating plant grains, plant reproductive organs, and fruits (Valente *et al*. 2013; Stotz *et al*. 2009a; Stotz *et al*. 2009b; Kovalchuk *et al*. 2010). In addition, some members of plant defensins show important activities for medical applications, including protease inhibitor (De Beer and Vivier, 2011), anti-cancer agents (Lin *et al*. 2010), and insecticidal activity (De Beer and Vivier, 2011).

Previous studies reported that defensins genes are arranged in highly polymorphic multigene families (Thomma *et al*. 2002; Schutte *et al*. 2002; Fedorova *et al*. 2002; Mergaert *et al*. 2003; Graham *et al*. 2004). Analysis of the sequenced plant genomes revealed that defensin genes are arranged in multigene families and are overrepresented in the genome of some plants species. Sixteen genes were found in the defensin gene family in *Medicago trancatula* (Hanks *et al*. 2005), whereas a family of 317 genes were detected in the defensins-like (DEFL) gene family in *Arabidopsis thaliana* (Silverstein *et al*. 2005).

Expression of plant defensin genes is induced by biotic during seed germination, flowering, and abiotic factors, but generally they have constitutive expression pattern (Padovan *et al*. 2010; Kovalchuk *et al*. 2010; Bahramnejad *et al*. 2009). Some plant defensins showed tissue-specific and developmentally regulated expression. Two examples were demonstrated for the defensins from tomato and maize that play a role during pollination (Stotz *et al*. 2009a; Amien *et al*. 2010). Lay *et al*. (2003) tested the expression of NaD1 defensin in ornamental tobacco (*Nicotiana alata*) and found that it was expressed in young flowers and roots.

The expression of defensin genes is not studied in many economic plants during various developmental stages. In wheat, there are 30 isolated cDNAs of defensins deposited in the nucleotide database (http://www.ncbi.nlm.nih.gov). In this study, the expression of three defensins genes was investigated during the germination of wheat grains. Results of this study would help in the characterization of the defense mechanism of Egyptian wheat varieties by expression of defensins which could contribute positively in the wheat breeding programs.

MATERIALS AND METHODS

Wheat varieties

Wheat grains of seven commercial wheat varieties were obtained from Egyptian Ministry of Agriculture (Table 1). They were grown in agar-water (0.5% agar) and kept in the dark for 7 days. Plant samples were collected and frozen at -80ºC or lypholysed (freeze-dried), ground into fine powder in a coffee grinder and stored at -80ºC until used for RNA isolation.

Table 1 . Egyptian wheat (*Triticum aestivum*) varieties used in this study.

NO	Variety	Status	Characteristics
1	Misr1	New	High tillers, resistant to rusts (stem rust in particular), 13.9%Protein, yield is 25 Ardab⁄Fed.
2	Giza168	Commercial	Resistant to three rusts, heat and drought tolerant, 12%protein, 21 Ardab⁄Fed.
3	Sakha94	Commercial	Resistant to three rust diseases, 11.5% Protein, 25 Ardab⁄Fed.
4	Sids 1	Commercial , (Upper Egypt)	Resistant to yellow but susceptible to leaf rust, heat tolerant, salinity tolerant, 13.2% protein, 19 Ardab⁄Fed.
5	Gemmiza 7	Commercial	Resistant to yellow and stem rust, 12.9% protein, 20 Ardab⁄Fed.
6	Gemmiza 11	Commercial	Resistant to three rust diseases, 12.2% Protein, 28 Ardab⁄Fed.
7	Shandawel1	Commercial	High adaptation and resistant to the three rust diseases, 12.6% protein, 27 Ardab⁄Fed.

Primer design

Full length mRNA for selected wheat defensin accessions from the Nucleotide database (http://www.ncbi.nlm.nih.gov) were used to design DNA primers using software on the website of Macrogen company (http://dna.macrogen.com). Primers were ordered from the same company. The Different designed primers for this study are summarized in Table 2.

Table 2: DNA primer sequence designed for cDNA synthesis and PCR amplification.

Primer	Sequence 5' → 3	Accession #	PCR Product, bp
PDF3	F-aaatggagttcaagcccaag R-agcgcctgattttgcagtag	KJ551518.1	225
PDF5	F- cccacagaagcaaatggagt R- agcgcctgattttgcagtag	KJ551520.1	236
PDF30	F- cctcctcctgctgctagttg R- tcattgccatccacgtttta	KJ551545.1	230
HvActin	F-tgccaagaacagctcctcag R-gaagcacttcctgtggacga	AY145451.1	460
PolyT	TTTTTTTTTTTTTTTTTTTTT		

RNA isolation

RNA was isolated from the collected wheat samples. One ml of QIAzol (QIAGEN Inc., Valencia, CA) was added to 10 mg of lyophilized powder and mixed. A volume of 0.3 ml chloroform was added to the homogenate. The mixture was then shaken for 30 s followed by centrifugation at 4°C and 13000 rpm for 20 min. The supernatant was transferred to a new tube. One fourth volume of 12 M lithium chloride was added and kept at -20°C overnight. Samples were centrifuged for 15 min at 4°C and 13000 rpm. RNA pellet was washed with 70% ethanol, briefly dried, and dissolved in DEPC water. The integrity of RNA was checked by agarose gel electrophoresis and its concentration and purity were determined at 260 nm and the OD260/280 ratio.

Synthesis of cDNA

A mixture of 2 µg of total RNA and 0.5 ng oligodT primer in a total volume of 11 µl sterilized DEPC- water was incubated in the Multigene thermal Cycler (Labnet, USA) at 65°C for 10 min for denaturation. Then, 4 µl of 5X RT-buffer, 2 µl of 10 µM dNTPs and 100 U M-MuLV Reverse Transcriptase (SibEnzyme Ltd. AK, Novosibirsk, Russia) were added and the total volume was brought to 20 µl by DEPC water. The mixture was then re-incubated in the thermal Cycler at 37°C for 1h, then at 90°C for 10 min to inactivate the enzyme then, hold at 4°C. cDNA was kept at -20°C until used.

Semi-quantitative PCR

PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl of 10 picomol of each primer (forward and reverse), and 12.5 µl PCR master mix (Promega Corporation, Madison, WI, USA). PCR was carried out using cycle sequence of denaturing at 94°C for 5 minute for one cycle, followed by 20 cycles which consisted of denaturation at 94°C for one minute, annealing at 55°C for 40 s, and extension at 72°C for one minute with additional cycle as a final extension at 72°C for 5 minutes. As a reference, expression of β-actin mRNA was tested using specific primers (Table 2). PCR products were electrophoresed on 2% agarose gel in TBE buffer at 100 volt for 50 minutes with ethidium bromide staining. PCR products were visualized under UV light and photographed. Densitometer analysis of band intensities was determined using NIH image program (http://rsb.info.nih.gov/nih-imageJ).

RESULTS

Total RNA isolated from wheat tissues was used for cDNA synthesis that was used as a template for PCR amplification. Three specific pairs of primers (Table 2) designed for three wheat defensin genes (PDF3, PDF5, and PDF30) were used to estimate the gene expression during wheat germination to test their natural expression. Specific primers for wheat defensin PDF3 gene amplified the expected fragment of 225 bp (Table 2) in all seven wheat varieties (Figure 1a). The expression of B-actin was estimated as a reference constitutive expressed gene (Figure 1b).

Table 3: Summary of gene expression level of wheat defensin genes estimated in this study.

Varieties	PDF3	PDF5	PDF30
Misr1	+	+	++
Giza168	++	++	++++
Sakha94	+++++	+++++	++
Sids1	+++	-	+++++
Gemmiza7	+++++	+++	-
Gemmiza11	++	++	++++
Shandawel1	+++++	+++++	++++

+ : 5000-10000 level of expression **++ : 10000-15000 level of expression**
+++ : 15000-20000 level of expression **++++ : 20000-220000 level of expression**
+++++ : >25000 level of expression **+, ++ = Low expression**
+++ = Moderate expression **++++, +++++ = High expression**

DISCUSSION

Gene expression of three wheat defensin genes was studied in seven Egyptain commercial wheat varieties at the germination stage using three pairs of specific primers. It was reported that defensin genes are highly expressed in grains during germination, roots, and floral buds ((Lay *et al*. 2003; Silverstein *et al*. 2005) because these tissues are highly protected against pathogens. The three primer pairs used in this study amplified PCR products of the expected size (Figure 1-3). Only one primer pair specific to PDF3 gene amplified products in all seven varieties (Figure 1). On the other hand, some primers did not amplify products in certain plant varieties (Figure 2,3). The primer pair that specific to PDF5 amplified products in 6 varieties, whereas it did not amplify product in Sids1(Figure 2). This indicates that this gene is not present or not expressed in this variety during seed germination, but this gene could be very essential and highly expressed in other developmental stages. The primer pair that specific to PDF30 gene did not amplify products in Gemmiza7 variety (Figure 3).

Absence of expression of some defensin genes in some varieties could be explained by the differential presence of defensin genes in the wheat defensin multigene family in different varieties or by their tissue or stage specific expression. Organization of defensin genes in multigene families was reported in previous studies (Thomma *et al*. 2002; Schutte *et al*. 2002; Fedorova *et al*. 2002; Mergaert *et al*. 2003; Graham *et al*. 2004). Hanks *et al*. (2005) studied the defensin gene family in *Medicago trancatula* and found that it contains sixteen genes. These genes were found to be expressed in different tissues including, leaves, flowers, grains, and roots, and their expression was differentially induced by various biotic and abiotic stimuli (Hanks *et al*. 2005). Silverstein *et al*. (2005) detected a family of 317 defensin-like (DEFL) genes in *Arabidopsis thaliana*. They were distributed into 16 clusters on five chromosomes. The expression of many of these DEFLs was tested and found to be expressed only in floral buds. In the current study, some members of the wheat defensin gene family (30 genes) might not exist in some varieties; therefore they give negative results with their specific primers (Fig. 2, 3). Sids1 gave negative results with PDF5 and Gemmiza7 gave negative results with PDF30. Another explanation for the differential amplification of wheat defensin genes could be due to that some gene members of this family might be expressed in a tissue-specific manner. Differential gene expression in different varieties during seed germination was found in this study. This could be explained as seed germination does not require the whole multigene family to work, but a combination of some genes is required at each stage of plant life cycle.

Results indicated that there was a differential gene expression in the same tissue. This is clearly evident since there was a difference in the expression level among species of the same gene during seed germination (Figure 1-3). Similar defensin expression pattern was reported in several previous studies (Beer and Vivier 2011; Lay *et al*. 2003; Hanks *et al*. 2005; Silverstein *et al*. 2005). In one example, four plant defensin genes (Hc-AFP1-

4) were isolated and studied from the native South African species *Heliophila coronopifolia* (Beer and Vivier 2011). Results of this study showed differential expression in the native plant as Hc-AFP1 and 3 were expressed in mature leaves, stems and flowers, whereas Hc-AFP2 and 4 were expressed in seedpods and grains (Beer and Vivier 2011). The expression of NaD1 defensin was estimated by In situ RNA blot analysis (Lay *et al*. 2003). A message of 0.6 kb of NaD1 transcript was detected in anthers, ovaries, pistils, and petals from ornamental tobacco (*Nicotiana alata*). At gene expression level, NaD1 transcript was not detected in leaves, whereas it was expressed at low level in roots, whereas it was highly expressed in younger flowers compared to mature ones (Lay *et al*. 2003). Tissue specific expression in the current study agrees with previous results (Silverstein *et al*. 2005; Lay *et al*. 2003) in that there was a difference in wheat defensin gene expression at germination stage among wheat varieties.

It is noteworthy that the commercial wheat varieties used in this study differ in their resistance to rust diseases, drought, and salinity (Table 1). These differences could be a reason for their differential defensins gene expression among the studied genes. Sids1 is susceptible to leaf rust, whereas Shandawel1 is resistant to the three rust diseases (Table 1). It is well known that defensins work as antimicrobial agents and contribute to resistance to abiotic stresses (Hanks *et al*. 2005).

Results obtained from this study will contribute to the plant biotechnology in two main aspects. First, evaluation of economic plant varieties for their defensins gene expression as a biodiversity tool and integrate the results in the breeding programs. Second, defensins genes would be used to genetically manipulate plants to enhance their resistance to biotic and abiotic stresses.

The quantitative estimation of defensin genes was measured with Image J software (Figure 1c). The quantitative estimation of the resulted bands revealed major variations in expression of this gene during wheat seed germination. Shandawel1 showed the highest gene expression level, while Sakha94 and Gemmiza7 indicated high expression. Sids1 gave moderate expression level, whereas Giza168 and Gemmiza11 showed low expression. Misr1 showed the lowest gene expression level (Figure 1c)

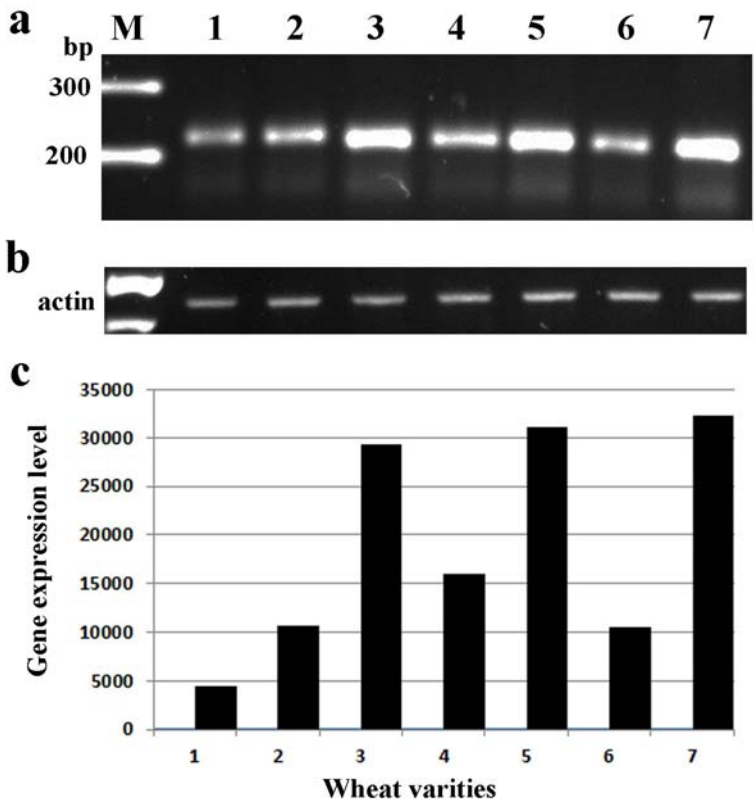

Figure 1: a. Agarose gel electrophoresis of RT-PCR products of wheat defensins using PDF3 primer: 1: Misr1, 2: Giza168, 3: Sakha94, 4: Sids1, 5: Gemmiza7, 6: Gemmiza11, 7: Shandawel1. b. β-actin gene expression. c. quantitative estimation of gene expression in a.

To estimate the expression level of PDF5 gene a pair of specific primers were used to amplify its cDNA. They amplified a 236 bp fragment in five wheat varieties i.e., Misr1, Giza168, Sakha94, Gemmiza7, Gemmiza11, Shandawel1, whereas this fragment was not amplified in Sids1 (Figure2a). This could explain the tissue specificity of the different members of wheat defensins gene family. Quantitative analysis of expression of PDF5 gene showed that Sakha94 had the highest gene expression level, whereas Misr1 showed the lowest gene expression level. Giza168 and Gemmiza11 showed low level of expression of PDF5 gene. Gemmiza7 showed moderate level of expression and Sandawel1 gave high expression (Figure 2c). The expression of B-actin was quantified as a reference constitutive expressed gene (Figure 2b).

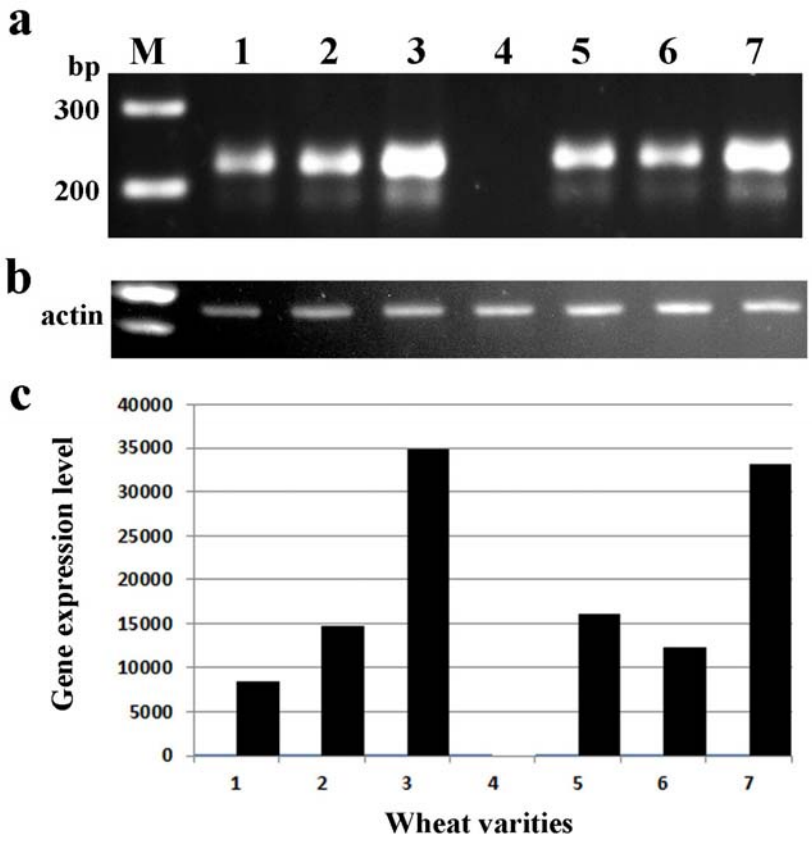

Figure 2: a. agarose gel electrophoresis of RT-PCR products of wheat defensins using PDF5 primer: 1: Misr1, 2: Giza168, 3: Sakha94, 4: Sids1, 5: Gemmiza7, 6: Gemmiza11, 7: Shandawel1. b. β-actin gene expression. c. quantitative estimation of gene expression in a.

When specific primers for PDF30 defensin gene were used to amplify its cDNA they also showed various level of expression. Its expression was not detected in Gemmiza7, while it was detected in the other 6 varieties (Figure 3a). Sids1 showed the highest expression. Giza168, Gemmiza11, and Shandawel1 indicated high expression level. Sakha94 showed moderate level of expression and Misr1 gave the lowest gene expression level (Figure 3c). The expression of B-actin was used as a reference constitutive expressed gene (Figure 3b).

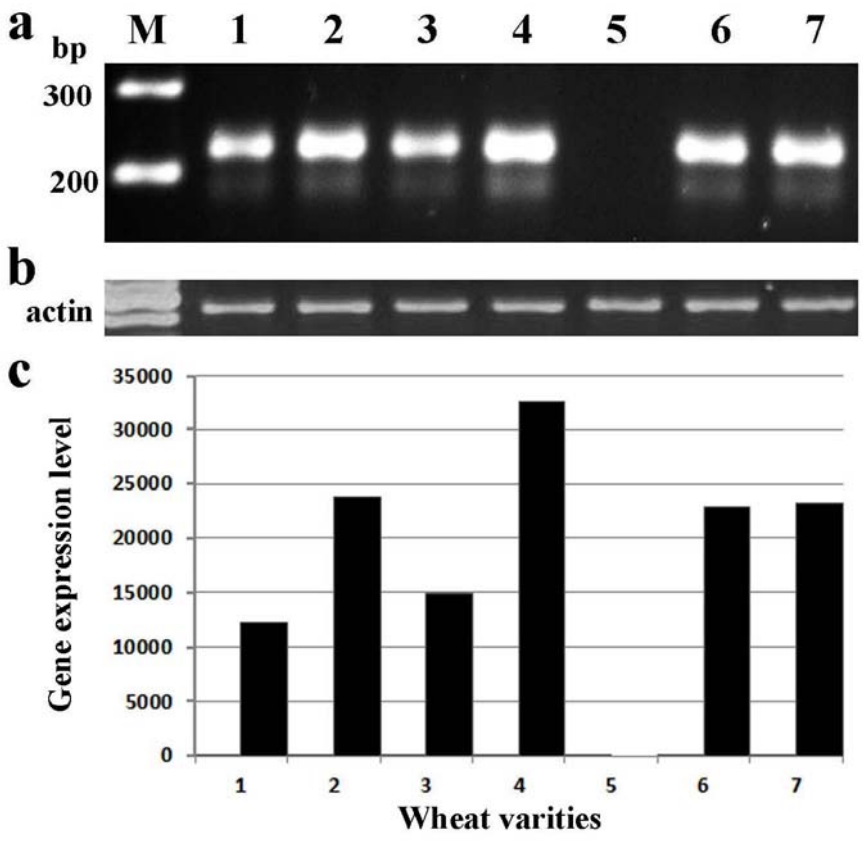

Figure 3: a. Agarose gel electrophoresis of RT-PCR products of wheat defensins using PDF30 primer: 1: Misr1, 2: Giza168, 3: Sakha94, 4: Sids1, 5: Gemmiza7, 6: Gemmiza11, 7: Shandawel1. b. β-actin gene expression. c. quantitative estimation of gene expression in a.

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تعبير جينات الدفينسين في أصناف القمح المصري *(Triticum aestivum)* أثناء إنبات الحبوب

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في هذه الدراسة تم إستخدام سبعة أصناف مصرية تجارية من القمح وهى مصر١، جيزة١٦٨، سخا٩٤، سدس١، جميزة٧، جميزة١١، شندويل١ لدراسة تعبير جينات الدفينسين أثناء إنبات البذور، حيث تم تصميم ثلاثة أزواج من البادئات المتخصصة لثلاثة من جينات الدفينسين في القمح (PDF3, PDF5, PDF30)، وتم إستخدام البادئات العكسية لتخليق جزيئات cDNA من RNA الكلي المستخلص من بادرات القمح متبوعا بإستخدام تفاعل PCR لإكثار شظايا المادة الوراثية DNA من جينات الدفينسين الأصناف السبعة.

أظهرت نتائج الدراسة أنه يوجد إختلاف في تعبير جينات الدفينسين الثلاثة المدروسة بين أصناف القمح السبعة تحت الدراسة، وتضمن ذلك غياب تعبير جين PDF5 في صنف سدس١ وغياب تعبير الجين PDF30 في الصنف جميزة ٧، وكذلك وجدت إختلافات في تعبير الجينات الثلاثة بين أصناف القمح السبعة المدروسة، حيث أظهر الصنف مصر١ أقل مستوي من التعبير الجيني للثلاثة جينات بينما أظهر الصنف شندويل١ أعلي مستوي من التعبير الجيني للجينات الثلاثة، أيضا وجدت إختلافات بين باقي الأنواع في تعبير كل جين من الجينات الثلاثة. يبدو من نتائج هذه الدراسة أن الإختلافات في تعبير جينات الدفينسين لها علاقة بمقاومة الأصناف للامراض والعوامل الغير حيوية. نتائج هذه الدراسة يمكن أن تساهم في إستخدام تعبير جينات الدفينسين كألية لقياس التنوع الحيوي في برامج تربية القمح وتقييم الأصناف.