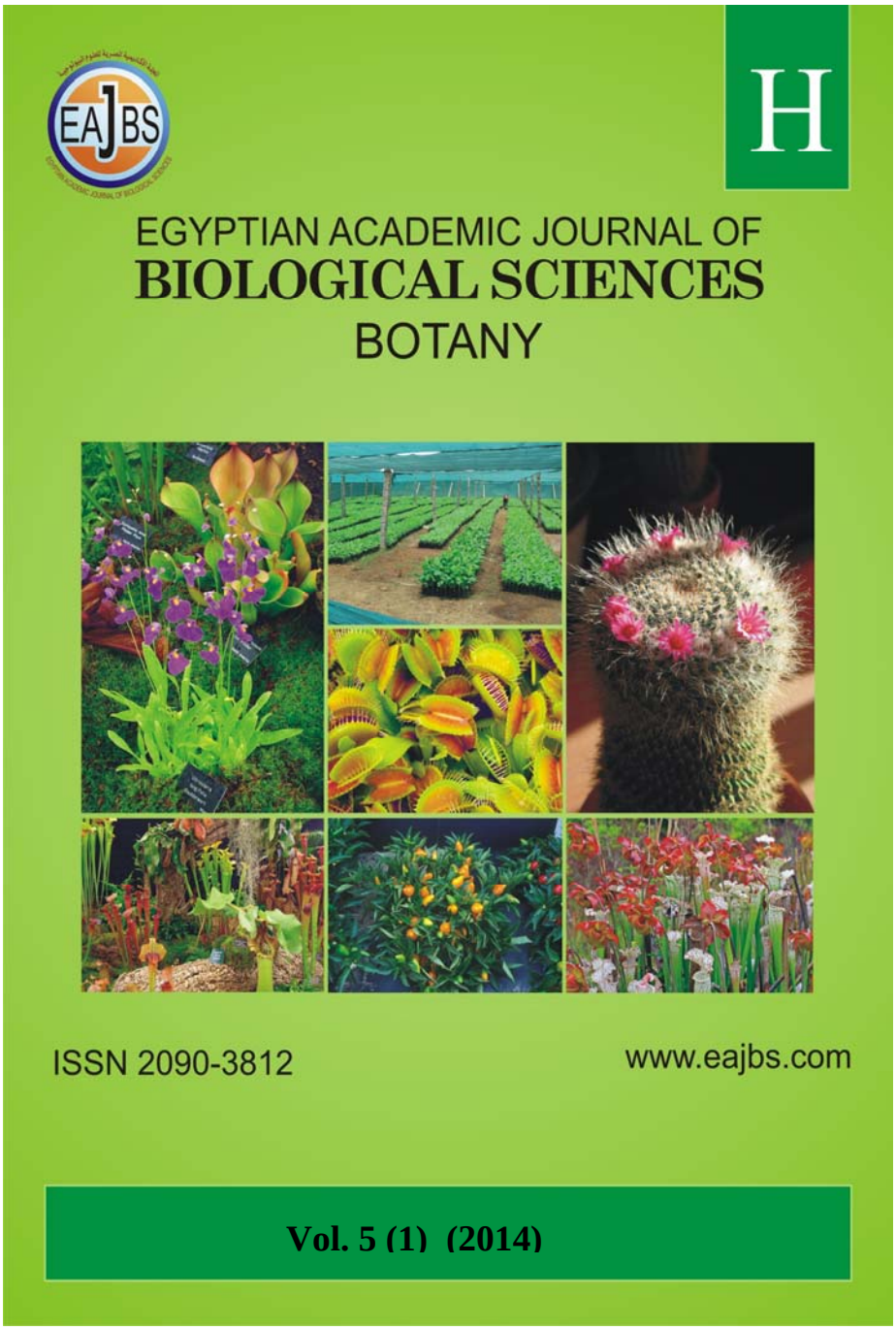

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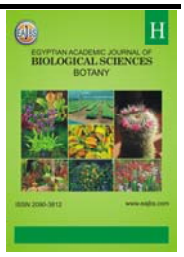

Determination of*TaGSK1* gene expression in selected wheat under different salt stresses

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ABSTRACT

TaGSK1 gene was shown to be differentially expressed in salt tolerant wheat plants. This study was conducted to reveal the expression of this gene in four selected wheat cultivated in Iraq. Two wheat cultivars (Dijila and Furat) and one genotype (2H) were grown in three salt levels, 0, 15, 25 ds/m. Meanwhile, cultivar (Tamooze 2) was grown only at zero level salt. Total RNA of the four plants was isolated, cDNA was synthesyzed and PCR amplification was tried. The results showed that a fragment of 189 bp was amplified in the selected salt tolerant wheat but not amplified in control one. In addition, *TaGSK1* gene was induced in salt tolerant wheat only and its expression level increased with increasing salt concentration as determined by SYBR green real time PCR. These results indicated the suitablility of *TaGSK1* gene as salt-tolerance marker in wheat under study.

Keywords: PCR; plant; salt, *TaGSK1*; wheat.

INTRODUCTION

Salt stress is a serious environmental factor limiting crop productivity and affects about 20% of irrigated agricultural land (Flowers and Yeo, 1995). Genes that limit the rate of salt uptake from the soil and the transport of salt throughout the plant, that adjust the ionic and osmotic balance of cells in roots and shoots, and that regulate leaf development and the onset of senescence are considered resbonsible of salinity tolerance (Munns, 2005). RNA finger-printing technique, cDNA-amplified fragment length polymorphism (AFLP) were used to analyze genes that are differentially expressed in both salt tolerance and sensitive with or without salt-stress (Lui *et al.*, 2000). Many genes encoding protein kinases have been shown to be induced under high NaCl conditions (Mizoguchi *et al.*, 1996). *TaGSK1* (*Triticum asetium* L. glycogen synthase kinase–shaggy kinase (*TaGSK1*)) gene derived from the genome of wheat salt-tolerance mutant RH8706-49 was studied. Its was induced by NaCl stress and its expression level was more strongly in salt-tolerant mutant than salt-sensitive genotypes as indicated by Northern blot, suggesting it may be involved in signal transduction of salt-stress in wheat (Chen *et al.*, 2003). In addition, *TaGSK1* expression was used as salt tolerance marker in wheat (Bahrami *et al.*, 2009). The aim

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of this study is estimate the *TaGSK1* gene expression using SYBR real time PCR for some selected wheat cultivated in Iraq.

MATERIALS AND METHODS

Wheat genotypes cultivation:

Three wheat cultivars and one genotype cultivated in Iraq were used in this research. Seeds of the four wheat plants were washed with tap water for 30 min, immersed in 50% of sodium hypochlorite then treated with 2-3 drops of Tween 20 for 10 min, finally these seeds were washed once with 70% ethanol and several times with distilled sterile water. From each plant, five sterilized seeds were placed in culture bottle containing 15 ml of solidified MS medium with each of 0 ds/m, 15 ds/m and 25 ds/m of salt concentrations (NaCl) without any growth regulators. Each treatment for wheat was replicated thrice. All cultures were performed in 16: 8 (L: D) photoperiod at 25^{o}C. Growth results were recorded after 15 days and leaves were taken for next steps.

RNA isolation and cDNA synthesis

Total RNA were isolated using Geneaid total RNA purification mini kit (Taiwan) accordingto the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I (Biobasic, Canada) for 20 min at 37°C, DNase I was inactivated at 65°C for 10 min. The integrity of the RNA was verified after separation by electrophoresis on a 1.5% agarose gel containing 0.5% (v/v) ethidium bromide (How do you validate RNA integrity and purity? PLZ specify). First-strand cDNA was synthesized from 500 ng of total RNA using Reverse Transcription System (Bioneer, Korea) with an oligo-dT_{15} primer. Reaction solution was used as template for reverse transcriptase polymerase chain reaction (RT-PCR) of the four wheat plants.

***TaGSK1* gene amplification**

TaGSK1 (target gene) and wheat housekeeping B-actin (reference gene) cDNA were amplified using primers in Table (1). Polymerase chain reaction was initiated with hot start method using the cDNA template on Labnet Thermo cycler (USA). The PCR reaction was carried out at 95°C for 5 min and 40 cycles at 95°C for 1 min, 60 °C for 45 s and 72°C for 1 min.

Table 1: Primers used for amplification of TaGSK1 and actin cDNA sequences.

Gene	Primer	Sequence5'-3'	Genbank accession number	Reference
TaGSK1	Forward	GTTTGGTCTGCTGGCTGTGTTCTT	DQ443471	(Guang *et al*., 2011)
	reverse	GTGCCATGGGTGAGCTTTGATTT		
Actin	Forward	TGGCACCCGAGGAGCACCCTG	AF326781.1	(Guang *et al*., 2011)
	Reverse	GCGACGTACATGGCAGGAACA		

Gene expression analysis by SYBR green real-time RT-PCR

The expression of *TaGSK1* gene was examined by SYBR real-time RT-PCR using Exicycler real time PCR (Bioneer, Korea). One step RT-PCR was performed using premix RT-PCR qPCR kit (Bioneer, Korea), following the manufacturer's protocol. The thermal cycling profile consisted of initial denaturation at 95°C for 5 min and 40 cycles at 95°C for 1 min, 60°C for 45 s and 72 °C for 1 min, followed with melting curve analysis at 60-95°C. To confirm amplification specificity, the amplified fragments were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide. Quantitation of relative expression was determined by the $2^{-(\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001). Each sample was run in triplicate. For

estimation of standard curve, online software was used to convert DNA concentration to log copy number (http://www.uri.edu/research/gsc/resources/cndna.html).

Statistical analyses:

Statistical analysis was carried out using MINITAB 11 (Minitab, Inc., Pennsylvania, USA).Analysis of variance (ANOVA) as shown in Tables (6 and 7) was employed to investigate significant differences in the TaGSK1 gene expressions under different NaCl concentrations treatment. Paired t test was calculated to determine the significant differences in the gene expression profile between wheat cultivars (Ramezani 2012).

RESULTS

The results outlined in Table (2) showed that salinity led to a significant decrease in the percentage of germination in Tamooze 2 cultivar. Results also indicated that there was a clear difference among wheat in the percentage of germination and particularly in the second and third levels (Clarify). This indicated that the (Furat and Dijila) cultivars and 2H genotype were more salt-tolerant than the (Tamooze 2) cultivar.

Table 2:Percentage of wheat germinationindifferent salt levels.

Cultivars	The percentage of germination in different levels of salt (ds/m)			rate
	0	15	25	
Furat	100%	100%	100%	100%
Dijila	100%	96%	89%	95%
2H	100%	94%	86%	93%
Tamooze 2	100%	13%	0%	37.6%
Rate	100%	75.75%	68.75%	81.5%

Total RNA was isolated, genomic DNA digested with DNaseI and the integrity were estimated by agarose electrophoresis (Fig. 1).

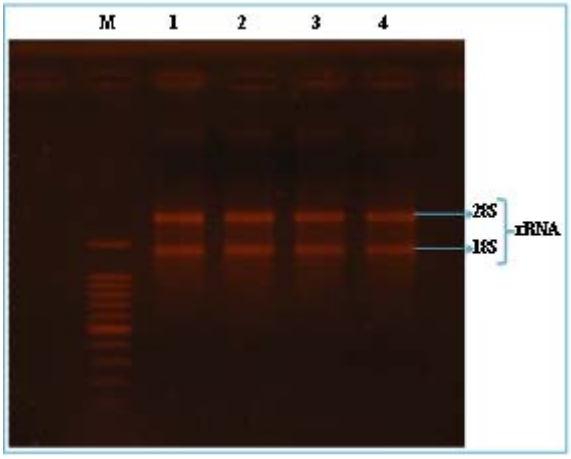

Fig. 1: Ethidium bromide stained agarose electrophoresis (1.5 %) of RNA isolated from wheat. M: Marker (100bp). 1: Furate cultivar, 2: Dijila cultivar, 3: 2H genotype, 4: Tamooze 2 cultivar.

RT-PCR results showed that the size of B-actin band was 118 bp and for *TaGSK1* was 189 bp (Figs. 2 and 3). Conventional and SYBR green real time PCR

were used for amplification of both genes, *TaGSK1* and B-actin. Melting curve analysis was used to indicate the presence of target genes and lack of Primer dimer or other non-specific DNA amplifications (Fig. 3). Absolute and relative gene expression of the *TaGSK1* gene was estimated using standard curve and reference gene (B-actin) (Figs. 4 and 5).

Standard curve shows slope and R^2 values to determine the efficiency of PCR reaction for *TaGSK1* gene amplification (Figs.6 and 7).

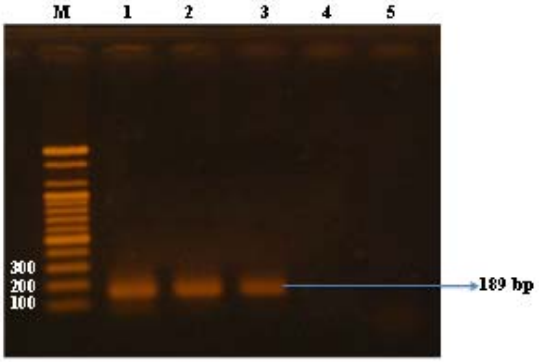

Fig. 2: Ethidium bromide stained agarose electrophoresis (1.5 %) of PCR product (*TaGSK1* gene) for wheat cultivars. M: Marker (100bp), 1: Furate cultivar, 2: Dijila cultivar, 3: 2H genotype, 4: Tamooze 2 cultivar, 5: Negative control.

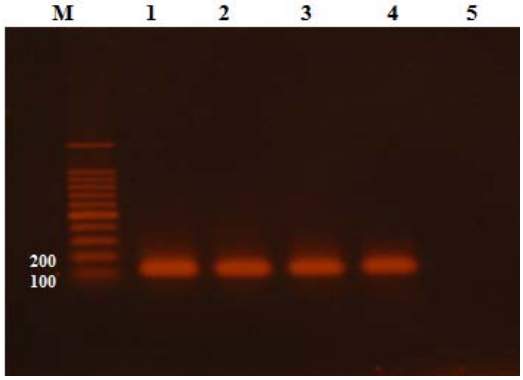

Fig. 3: Ethidium bromide stained agarose electrophoresis (1.5 %) of PCR product (B-actin gene) for wheat cultivars. M: Marker (100bp), 1: Furat cultivar, 2: Dijila cultivar, 3: 2H genotype, 4: Tamooze 2 cultivar, 5: Negative control.

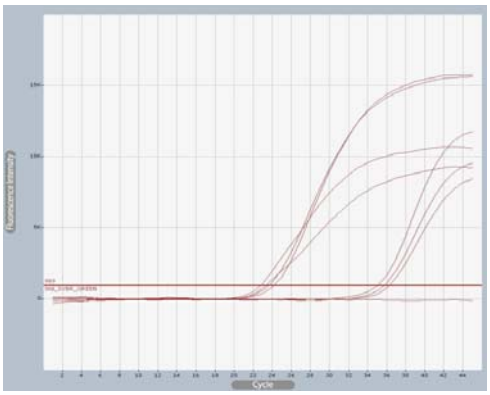

Fig. 4: SYBR Real time PCR amplification curves for *TaGSK1* gene and B-actin gene of all wheat (Furat, Dijila, Tamooze 2 cultivars and 2H genotype).

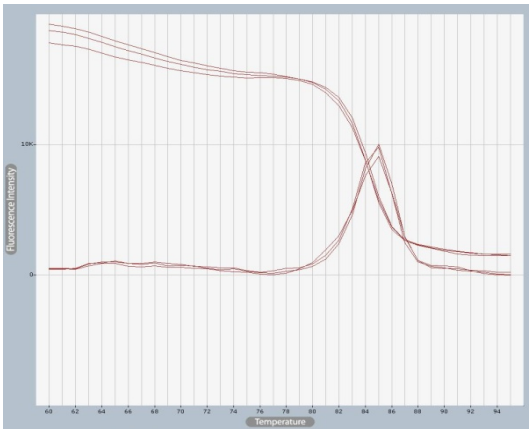

Fig. 5: Derivative and raw melting curves of *TaGSK1* gene for cultivars Furat, Dijila, 2H.

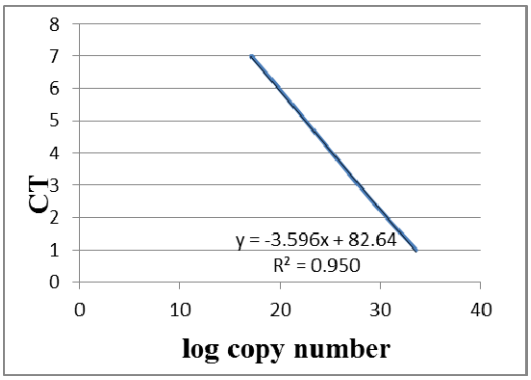

Fig. 6: Standard curve shows slope and R^2 values to determine the efficiency of PCR reaction for *TaGSK1* gene amplification.

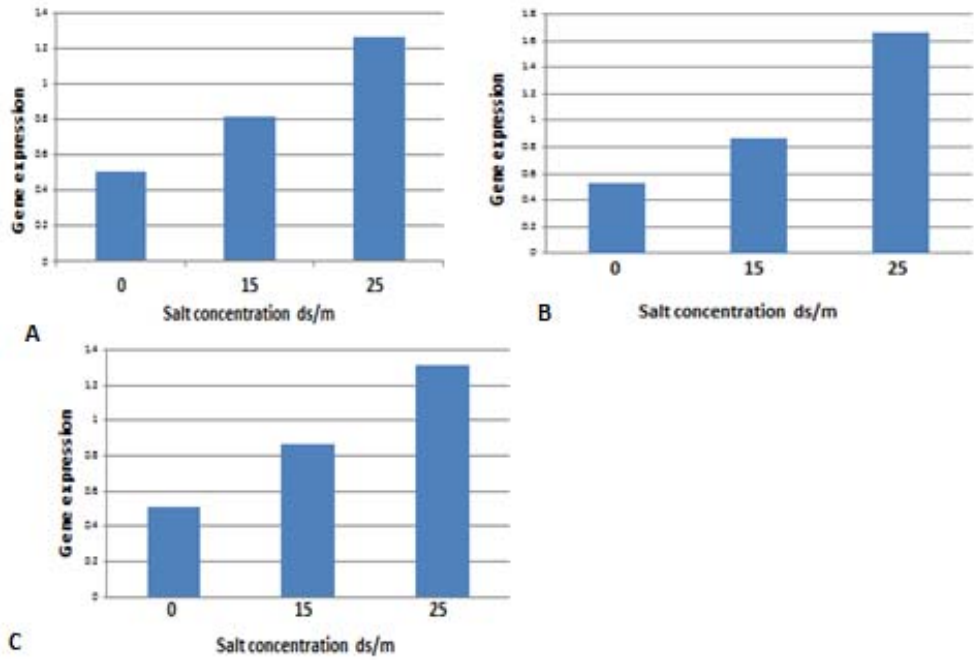

Fig. 7: TaGSK1 gene expression under different NaCl concentrations (0, 15 and 25 ds/m); A)Furat, B) Dijla, C) 2H genotypes.

Tables (3 and 5) listed the values of CT for B-actin and *TaGSK1* genes and referred to the rate of gene expression for each concentration of saline and each sample of wheat studied.

Table 3: CT values of B-actin and *TaGSK1* genes SYBR green real time PCR amplification.

Cultivars	Salt concentration ds/m	CT value of B-actin	CT value of *TaGSK1*
Furat	0	23.1	29.4116
	15	23.09	24.92665
	25	23.02	20.84625
Dijila	0	23.125	29.02525
	15	23.36	24.6535
	25	23.96	19.31815
2H	0	23.95	30.12365
	15	23.96	24.90595
	25	23.97	21.432
Tamooze 2	0	0	0
	15	------	-------
	25	-----	-------

In order to study gene expression, the efficiency of RT-qPCR was determined by using several serial decimal dilutions of eluted PCR bands. The logarithm of gene copy numbers were estimated according to DNA concentrations using the online software DNA copy number (Table, 4):

Table 4: CT values and the number of copies of the DNA template of the gene *TaGSK1*

CT values	Log copynumber
23.11	1.19033
22.13	2.29033
21.34	4.29033
20.43	8.11033
19.56	13.72033
18.66	15.81033

Table 5: Values of *TaGSK1* gene expression.

Wheat	Salt concentration ds/m	CT(*TaGSK1*) – CT (*Actin*)	Gene expression= $E^{[Ct\ (TaGSK1) - Ct\ (Actin)]}$
Furat	0	6.3116	0.5035
	15	1.83665	0.8190
	25	-2.17375	1.2662
Dijila	0	5.90025	0.5265
	15	1.2935	0.8688
	25	-4.64185	1.656
2H	0	6.17365	0.5111
	15	1.30	0.8682
	25	-2.538	1.3176
Tamooze 2	0	0	0
	15	-----	-----
	25	------	------

Table 6: Anova one way test for the investigation of differences in GSK1 gene expression profiles under different NaCl concentration in each wheat cultivar (P = 0.05).

NaCl Concentration	FURAT	DIJLA	2H
0	0.4366*	0.5115*	0.5413*
15	0.7594*	0.8208*	0.9050*
25	1.2655*	1.5753*	1.0856*

*=Significance difference

Table 7: Paired t test compares GSK1 gene expression profiles in different wheat cultivars.

Wheat genotypes	GSK-1 gene	
	Mean	P value
Furat:Dijla	0.820: 0.969	0.73
Furat:2H	0.820:0.844	0.94
Dijla:2H	0.969: 0.844	0.76

DISCUSSION

There is an extensive evidence for the importance of phosphorylation and dephosphorylation in plant response to salinity stress (Xiong *et al.,* 2002). *TaGSK1*, a salt-inducible gene was previously shown by several researchers to involve in salt tolerance. Recently, this gene was cloned into *Arabidopsis* plants which enhanced salt tolerance (He X *et al.,* 2012). The results of this study showed that *TaGSK1* gene was expressed in the salt-tolerant cultivars (Dijila and Furat) and the genotype (2H) but not expressed in salt-sensitive cultivar (Tamooze 2). These results are in agreement with the results of (Chen *et al.,* 2003, Bahrami *et al.*, 2009). They indicated the importance of this gene as wheat salt-resistance marker.

The results also showed that *TaGSK1* gene expression increased with increase of salt concentration. These results indicated that *TaGSK1* gene is very important in salt tolerance as its expression incearesed with salt stress increase (Chen *et al.,* 2003: Bahrami *et al.*, 2009; Guang *et al.,* 2011; He *et al.,* 2012).

The result also indicated that B-actin gene was suitable as a reference gene for the used plant cultivars due to its stablity at specific CT value during SYBR green real time PCR in salt resistance and sensitive wheat plants.

In conclusion, *TaGSK1* gene can be used as a salt tolerance marker for Iraq local wheat plants.

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