DETERMINATION OF *ZMHKT1,5* GENE EXPRESSION UNDER DIFFERENT SALT STRESSES USING PLANT TISSUE CULTURE of MAIZE (*ZEA MAYS L.*).

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ABSTRACT

This study was designed to determine *ZmHKT1*;5 gene expression in relatively salt tolerant using four maize genotypes (Alfajr and Almaha and medium-tolerant ones T1 and T6) within cellular level. Three salinity levels (2, 8, and 16 ds / m . Were used immature seeds were surface sterilized and embryos were dissected under full sterilization conditions. Callus was initiated from immature embryos of the above four genotypes on Skoog and Murashige (MS) medium supplemented with the required growth regulators (3 mg/L 2,4-D +0.5 mg/L kinetin) and recultured for three times and transferred to the stress medium containing NaCl. K^+/Na^+ and Ca^+ / Na^+ were measured and *ZmHKT1*;5 gene expression was estimated using Real-time PCR in callus cultures after tree cultures. Results indicated that a significant decrease in calcium and potassium ion concentrations with increasing NaCl level. The highest concentration was recorded in the genotype Almaha, while the lowest was in Alfajr. The highest concentration of gene *ZmHKT1*;5 expression was achieved in the Alfajr genotype reached 5.59 at 16 ds/m.

Key words: genotypes, Salinity, K+/Na+ ratio, Ca^{++} / Na^+ ratio, gene expression, quantitative detection.

تحديد التعبير الجيني للجين ZmHKT1;5 في الذرة الصفراء بتقينة زراعة الانسجة النباتية تحت ظروف الاجهاد الملحي

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المستخلص

تهدف هذه الدراسة لتشخيص الجين المتحمل للملوحة *ZmHKT1*;5 ودراسة تعبيره الجيني باستخدام اربعة تراكيب وراثية من الذرة الصفراء (الفجر والمها المتحملة للملوحة والمتوسطة التحمل للملوحة T1و T6) ضمن ثلاث مستويات من الملوحة (2، 8 ،16) ديسيسمنز/م). عقمت البذور الغير ناضجة سطحيا ومن ثم تم استئصال الاجنة تحت ظروف التعقيم الكاملة. تم زراعة الاجنة وتحفيز الكالس عن طريق زراعتها في الوسط الغذائي (MS) الحاوية على منظمات النمو (2,4-D 3 ملغم/لتر + الكاينتين 5 ملغم/لتر) ومن ثم اعادة زراعتها ثلاث مرات ونقلها الى وسط ملحي حاوي على كلوريد الصوديوم . تم قياس نسبة الصوديوم /الكالسيوم ونسبة الصوديوم / البوتاسيوم ودراسة التعبير الجيني للجين *ZmHKT1*;5 بواسطة تقنيةReal-time PCR . اشارت النتائج حدوث انخفاض معنوي في ايون البوتاسيوم والكالسيوم بزيادة مستويات الملوحة. سجل التركيب الوراثي المها اعلى تركيز واقل منه الفجر. الجين *ZmHKT1*;5 اعطى تعبير جيني في التراكيز العالية من الملح حيث اعطى التركيب الوراثي الفجر تعبيرا 5.59 في المستوى الملحي 16 ديسسمنز/م).

كلمات مفتاحية: التراكيب الوراثية، الملوحة، K^+/Na^+، Ca^{++} / Na^+ ، التعبير الجيني، التشخيص الكمي.

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INTRODUCTION

Salt stress is one of the most important challenges facing agricultural production in many countries, especially in irrigated agricultural areas. Iraq is one of the countries that suffer from water scarcity in agriculture, in addition to the increasing problem of salinity due to the heaving irrigation and poor drainage systems, which have led to excessive accumulation of salts in soil (12). Wide areas of the Iraqi lands have already been affected by salinity, particularly in surface irrigated ones. This has negatively affected to the productivity of these lands, in terms of quantity and quality particularly maize crop. Maize is a crop of a medium sensitivity to tolerate salinity and because it is considered one of the important field crop in Iraqi and even other regions of the world. The expansion of cultivation and production of this crop in the soils affected by salts using traditional and conventional breeding methods is still viable but requires the introduction of new technologies to improve yield accompanied with good quality by adopting salt tolerant genotypes (18). One of the unconventional methods in raising this crop is the use of plant tissue culture technology to stimulate callus from dissected immature embryos on a defined medium then supplement with different levels of salt to select cells capable of dividing on high salt stress (11). Induction of callus allows the researcher to obtain large numbers of cells in small space like a Petri dish under controlled conditions, without the interference of the rest environmental (10). Several salinity-stimulating genes are also stimulated by salt stress, which indicate similar mechanisms of stress responses. These genes are classified into three main groups: 1/ Those encode products that protect plant cells directly from stress such as heat stress proteins (HSPs), LEA proteins, reverse osmosis, anti-freezing proteins, detoxification enzymes, and free radicals. 2/ those that participate in succession and reproduction control signals, such as mitogen-activated protein kinase (MAPK), a calcium-dependent protein kinase. 3/ those involved in the absorption and transport of water and ions such as aquaporin and ion transporters (7). Therefore, this study aimed to select cell lines from four corn genotypes and examination of gene expression, and thus recommending the best one to perform under salt stress.

MATERIALS AND METHODS

Four maize (*Zea may*s L.) genotypes grown in Iraq, namely, Alfajer, almaha, T1 and T6 were used in the current work. Seeds were collected from immature kernels and subjected to surface sterilization (17). Immature embryos were transferred aseptically into Murashige and Skoog medium (6) supplemented with 3 mg/l 2,4-D +0.5 mg/l kinetin and the pH was adjusted to 5.7 and autoclaved at 121°C for 15 minutes (19). Cultures embryos were incubated in dark at 22 ± 2 ◦C (2) . Initiated callus was transferred into NaCl concentrations incorporated in the medium after 4 recultures. K+/Na+ and Ca^{+} / Na^{+} and gene expression was measured in calli after 4 weeks growth in stressed medium. K, Na and Ca were measured using Flame photometer (5) . Total RNA were isolated using Geneaid total RNA purification kit (Taiwan) according to the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I (Biobasic, Canada) for 30 min at 37°C, DNase I was inactivated at 60°C for 10 min. The integrity of the RNA was verified after separation by electrophoresis on a 2% agarose gel containing 0.5% (v/v) ethidium bromide. First-strand cDNA was synthesized from 400 ng of total RNA using Reverse Transcription System (Bioneer, Korea) with an oligo-dt_{20} primer. Reaction solution was used as template for reverse transcriptase polymerase chain reaction (RT-PCR) for the four maize genotypes. ZmHKT1,5 (target gene) and maize housekeeping B-actin (reference gene) cDNA were amplified using primers , listed in Table 1. Polymerase chain reaction was initiated with hot start method using the cDNA template on bioneer Thermo cycler (korea). 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 *ZmHKT1,5*

Gene	Primer	Sequence5'-3'	Refrence
ZmHKT1;5	Forward	TCAACTTCAGCGTCCTCAACA	(9)
	reverse	GAATCCCACGTTGCCATACG	
Actin	Forward	TGGCACCCGAGGAGCACCCTG	
	Reverse	GCGACGTACATGGCAGGAACA	

The expression of *ZmHKT1*,5 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 manufacturers 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 60-94°C. Quantitation of relative expression was determined by the $2^{-(\Delta\Delta CT)}$ method (14).

RESULTS AND DISCUSSION

The results in Table 2 show a significant effect of salinity levels on the calcium and potassium ion concentrations. Their concentration decreased significantly, with increasing of the concentration of sodium chloride salt in the nutrient medium, recording the lowest concentrations (6.65, 5.73) respectively, while their highest concentrations were 16.9 and 53.5 respectively. The results revealed that the maize genotypes had a significant effect on the concentration of these two ions in callus tissue reaching their highest concentrations 12.1, and 28.7 in the genotype Almaha, while the first concentration of calcium was achieved in the genotype Alfajr recording 8.17, while the lowest concentration of calcium was achieved in the genotype Alfajr. The lowest concentration of potassium ion was achieved in Alfajr callus tissue (19.57). The results also revealed significant interactions among the genotypes and levels of salinity at the concentrations 4.3, 0.44 in callus tissues. The highest concentration of calcium ion was achieved in genotype (T1) which had 20.8. The highest concentration of potassium ion was found in the genotype Almaha at the NaCl level 64.5 which is differed significantly with most interactions.

Table 2. Effect of NaCl addition to MS medium on Ca^{++} / Na^{+} and K^{+} / Na^{+} in maize callus tissues

K^{+} / Na^{+}				Ca^{++} / Na^{+}				Genotype
	Salinity (EC) ds/ m				Salinity (EC) ds/m			
Means	16	8	2	Means	16	8	2	
19.57	12.5	16.13	30.1	8.17	8.6	8.8	7.13	Alfajr
28.76	9.2	12.6	64.5	12.1	8.2	8.5	19.6	Almaha
22.83	0.80	5.11	62.6	11.2	5.5	7.3	20.8	T1
20.39	0.44	3.94	56.8	10	4.3	5.8	20.1	T6
22.8	5.73	9.44	53.5	10.3	6.65	7.6	16.9	The mean

The results in Table 3 show a significant effect of salinity levels on the gene expression of the gene *ZmHKT1*,5. But the expression decreased significantly, with increasing in the concentration of sodium chloride and its lowest was 0.6293 in the genotype (T1). The results also indicated highest interactions among genotypes and salinity levels. The highest gene expression was revealed in the genotype Alfajr, which had 5.59 and differed significantly at the most interactions.=

Table 3. Effect of different concentrations of NaCl on the expression of ZmHKT1,5 gene in callus tissues in four maize genotypes

Genetic expression of the ZmHKT1; 5 gene				Genotype
Salinity (EC) ds/m				
16	8	2	Means	
5.5947	3.0314	1	3.2087	Alfajr
4.8398	3.4622	1	3.1	Alfajr
0.8034	1.4785	1	1.0939	T1
0.6293	1.2768	1	2.0930	T6
2.9668	2.3122	1	2.3	The means

The results show in Figure 1 *ZmHKT1*,5 gene amplification plots by using quantitative PCR (qPCR). The cycle threshold (Ct) values are invers to the amount of gene expression. Lower Ct value indicate high amount of gene expression while higher Ct value means lower or too litter amount of gene expression. Fig 1 indicate that the high sodium, calcium and potassium causes a high expression *ZmHKT1*,5 gene so that refers to the importance of this gene as maize salt resistance marker.

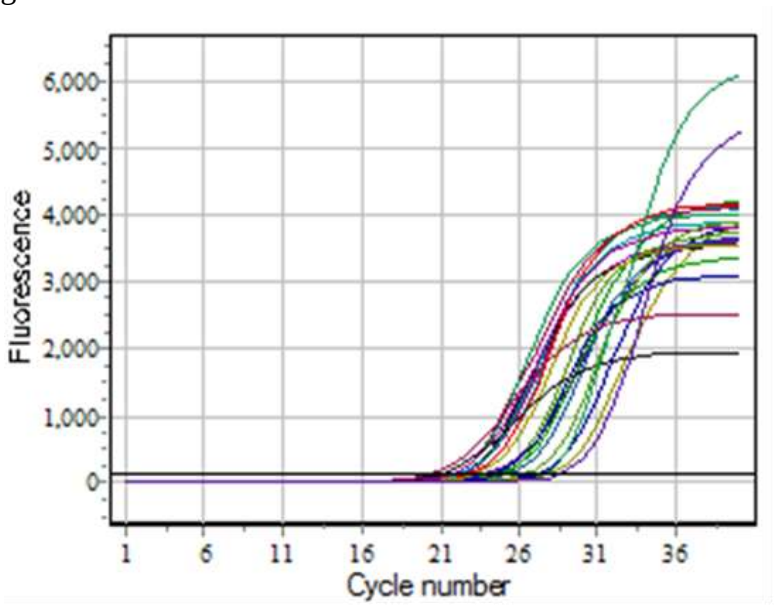

Figure 1. Real time gene reading

The expression of ZmHKT1,5 gene was examined by SYBR real-time RT-PCR using Exicycler real time PCR (Bioneer, Korea). RT-PCR-based real time is a preferred method of measurement because it is one of the most sensitive detection methods that provide real number and repeatable, multi-analysis in a large number of samples in a short time (15). Plant tissue culture could be used to assess the genotypes of maize to tolerate salinity and thus determine the most tolerant cell lines according to their ionic composition (20). Genes which control to salt tolerance could be overexpressed when cells are exposed to salinity (3,4). *ZmHKT1; 5*, 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. The results of this study showed that *ZmHKT1; 5* gene was expressed in the salt-tolerant cultivars (Alfajr and Almaha) and the comparison of the genotype (T1 and T6), but at different levels depending on the type of genotype .These results are in agreement with the results of other researchers (9,1). They indicated the importance of this gene as maize salt-resistance marker. The results also showed that *ZmHKT1; 5*gene expression increased with increasing the salt concentration. These results indicated that *ZmHKT1; 5* gene is very important in salt maize tolerance as its expression increased with salt stress increase (9, 13) .The result also indicated that B-actin gene was suitable as a reference gene for the used plant cultivars due to its stability at specific CT value during SYBR green real time PCR in salt resistance and sensitive maize plants (8,16) . In conclusion, *ZmHKT1; 5* gene could be used as a salt tolerance marker for some maize genotypes.

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