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Detection the Expression of two Genes *ZmWRKY86* and *PMP3* responsible for tolerance to salt stress in maize

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Abstract: The current study measured the expression of certain genes responsible for the tolerance of the maize (*Zea mays L.*) to salt stress when it was grown in soil containing (0.0,25, 50,75,100 mmol) sodium chloride (NaCl) for 30 days. The results indicated that the germination percentage decreased with the increased salt concentration. Isolated ribonucleic acid (RNA) from young leaves of maize seedlings was used as a template for manufacturing a piece of complementary nucleic acid (cDNA), gene expression was measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR) for both *PMP3* and *ZmWRKY86* genes responsible for salinity stress tolerance. The expression level of *the PMP3* gene increased with increasing salt concentration and reached 1.38 and 1 .86 at concentrations of 50 and 75 mmol, respectively, but decreased with increasing salt concentration to 100 mmol by 0.34%. The expression of the second gene *ZmWRKY86* was increased with increasing salt concentration and reached a maximum level of 3.18 at 100 mmol of NaCl.

Keywords: Maize, gene expression, salt tolerance, salt tolerant gene**.**

Introduction

Maize (*Zea Mays* L.) is one of the important crops for producing high-grain raw material in industrial products and is consumed as a staple food (Liu *et al*., 2022). All parts of corn are nutritionally beneficial as well as used in non-food products (Ahanger *et al*., 2020; Nawaz *et al*., 2020). Its seeds contain 10% protein, 72% starch, 3% sugar, 17 ashes, 4.8% oil, and 8.5% fibre (Noman *et al*., 2015; Mohammed *et.al*., 2023). Increasing maize productivity is a must due to the increasing population and the decreasing number of arable lands (Hansen *et al*., 2019).

In the past contracts, plants have suffered from many environmental threats to their growth and evolution, such as biotic stresses (resulting from pests and diseases) in addition to abiotic stresses resulting from drought, extreme temperatures, salinity, and the accumulation of heavy metals, etc. Salinity is one of the stresses that pose a serious danger to the agricultural economy (Ahmad *et al*., 2019; Ali *et al*., 2020). Recently, increase salinity has led to economic losses related to the crop, as environmental changes have led to an increase in the rate of evaporation due to rising temperatures and an increase in the flooding of

drylands due to the rise in seawater level, in addition to the uncontrolled exploitation of groundwater (Shahid *et al*., 2018). Estimates indicate that about 1.128 million acres of lands are affected by salinity around the world, with salinity affecting the maize farming in every part and at all stages of its growth (Alam *et al*., 2021). Salinity affects the germination of seeds by restraining water absorption due to the toxicity of Cl^- and Na^+, which changes the osmotic stress of the soil solution (Mohamed *et al*., 2020; Hassan *et al*., 2021; Olayinka *et al.,* 2023). The accumulation of salts in the soil also leads to the inhibition of plant growth and stunting, as well as the cessation of leaf expansion and internal growth due to the terminated cell growth and programmed cell death in the vegetative stage of the plant (Zaidi *et al*., 2022). The reproductive stage of the maize plant is affected by salinity due to the decreased rate of the photosynthesis process, which leads to an imbalance in the carbon required for plant growth, and the abortion of fertilised grains leads to a decrease in the percentage of grains and seed numbers (Ali *et al*., 2020). Developing salt tolerance in maize crops is an important practice to decrease salinity stress on the plant. There are many studies on salinity tolerance in maize crops, such as the genetic region QTLs, which is responsible for salt tolerance in maize (Luo *et al*., 2021). Additionally, many genes were discovered that play a key role in the tolerance of maize salinity stress and decrease its negative effects. *BSA* is one such gene (Qin *et al*., 2020). *BSA* was diagnosed in maize and is responsible for tolerating salinity stress (Zhu *et al*., 2023). The *BSK* gene has also been studied and found to be responsible for the plant's tolerance to abiotic stresses such as salinity, drought, and cold (Kang *et al*., 2021). The *ZmBski* gene identified in maize plants is responsible for drought tolerance (Liu *et al*., 2021). Plasma membrane protein (*PMP3*), a class of small hydrophobic peptides with small molecular weights and heterologous expression of PMP3 homologues (PMP3hs), is responsible for plant resistance to drought, salt, cold as well as abscisic acid. Hence, these peptides play important roles in maintaining ionic homeostasis (Kwok *et al*., 2020), in addition to proving that this gene is responsible for the tolerance of maize plants to salinity (Liu *et al*., 2022). WRKY factors are involved in various important processes in plants, including growth, sugar metabolism, defence, and stress responses. The overexpression of *WRKY114* in rice led to reduced salt stress tolerance and diminished sensitivity to abscisic acid (ABA) by affecting the expression of stress- and ABA-related genes (Bo *et al*., 2020). *ZmWRKY17*, when overexpressed in maize, resulted in increased sensitivity to salt stress while reducing sensitivity to ABA. This effect was achieved by regulating the expression of various ABA and stress-responsive genes. Furthermore, the overexpression of *the ZmWRKY17* mutant caused a reduced sensitivity to ABA during seed germination and early seedling growth (Li *et al*., 2023).

This study aimed to determine the expression of genes on the tolerance of salinity in maize plants.

Materials & Methods

Greenhouse Experiment

Maize seeds (a local variety) obtained from the Nineveh Agriculture Department were characterized by their genetic stability and high productivity. These seeds were grown in a pot containing soil treated with different levels of NaCl salt (25, 50, 75, and 100 mmol) alongside a control sample using salt-free soil with 3 replicates for each treatment. The plants were harvested one month after germination.

Molecular studies were conducted and the percentage of germination was calculated according to the following equation described by Hamidi *et al*. (2010).

$$Germination\% = \frac{germinated\ seeds}{total\ seeds} \text{ x } 100$$

Molecular experiment:

The ribonucleic acid (RNA) isolated from young leaves of maize seedlings was used as a template for manufacturing a piece of complementary nucleic acid (cDNA), whose nucleotide sequence is identical to the nucleotide sequence of the original gene that makes up the RNA using special primers that depend on the activity of the DNA polymerase enzyme.

Analysis of the level of genetic expression of the (*ZmWRKY8* and *PMP3)* genes by RT-PCR technology:

The process involves the quantitative assessment of the expression level of a gene (*ZmWRKY8* and *PMP3*) in maize plants through many steps:

RNA extraction:

After crushing the plant tissue taken from one-month-old young leaves of maize seedlings, it was mixed with 1 ml of Trizol, and then nucleic acid mRNA was extracted by the kit supplied by the Trans Company.

The process of converting the extracted mRNA molecule into a cDNA molecule:

After completing the mRNA extraction process, it was converted into a cDNA molecule depending on the effectiveness (reverse transcriptase) of the enzyme by using the kit supplied by Trans company. The random primer was prepared by maintaining it at 25ºC for 10 minutes and then adding an enzyme (Easy Script RT/RI Enzyme Mix) to the extracted RNA sample stored at 42ºC for 15 minutes for the purpose of conducting interactions of RT-PCR. The mixture was incubated at 85ºC for 5 seconds to inactivate the enzyme.

RT-PCR reaction

The quantitative testing of gene expression level used Gene-specific primers with primers for the housekeeping gene, with component RT-PCR reaction and program as shown in Tables (1, 2, 3). Livak ΔΔ method was depended to estimate gene expression.

Results & Discussion

Effect of salt stress (NaCl) on germination percentage:

Table (4) reflected a clear difference in the germination rate between the different salt concentrations. The germination percentage in the 25 mmol concentration reached 80% as compared to the control sample which reached 90%. In addition, the germination percentage decreased with the increase in salt concentration, reaching 70%, 50%, and 30% at respective concentrations of 50, 75 and 100 mmol.

Frequency of gene expression:

Gene expression was measured by RT-PCR for both *PMP3* and *ZmWRKY86* genes responsible for salinity stress tolerance of maize plant at four different NaCl salt concentrations of 25, 50, 75 and 100 mmol as well as a control sample (0.0 mmol). The cycle threshold is the baseline for the amount of gene expression in this technology measured by the one-step method in which (RNA) is converted to (cDNA) in one tube. The gene expression was measured by the Relative Quantification method and the ΔCT value between the target and housekeeping gene was determined for each treatment. The expression level of *the*

PMP3 gene decreased at NaCl salt concentrations of 25 mmol and it was 0.84 compared with control which it was 1 and increased with increasing salt concentration and reached 1.38 and 1.86 at concentrations of 50 and 75 mmol, respectively, but decreased with increasing salt concentration up to 100 mmol which it reached 0.34 These results can be explained by the fact that the dynamics of the gene expression response to salt stress is largely dependent on cell physiology (Sonsungsan *et al*., 2024), with severe osmotic stress at the threshold concentration leading to an increased delay in the activation of resistance genes to this stress (Park *et al*., 2020). The expression of the second gene, *ZmWRKY86*, increased with increasing salt concentration and reached a maximum level of 3.18 at 100 mmol of NaCl (Table 5& 6). Eight *ZmPMP3* genes have been identified in maize plants, encoding membrane proteins, most of which are able to maintain the membrane's function of regulating intracellular ionic homeostasis (Fu *et al*., 2012). It is likely that plant tolerance to osmotic stress was achieved by reducing the oxidative stress due to the possible involvement of *ZmPMP3-1* in regulating ionic homeostasis, and this may also suggest that these conserved small hydrophobic peptides could be an effective way to improve plant's salt tolerance. *PMP3* protein (*PmPp3p*) has also been identified in the yeast *Saccharomyces cerevisiae*, which functions in maintaining plasma membrane functions. Deletion of the gene encoding increased the sensitivity of yeast cells to Na^+ and K^+ and also led to an overexpression of these elements (Navarre *et al*., 2000). Another study suggests that *PMP3* genes are involved in maintaining the ionic homeostasis intracellular, however, because of their small size, they are likely not ion transporters (Zhang *et al*., 2008). While it is generally agreed that *PMP3* is involved in indirect cation uptake, its exact mechanism is currently unknown. The regulatory function of ion transporter genes in plants, such as *AVP1, AHA2* and *NHX1*, is likely to regulate ion homeostasis by the *ZmPMP3-1* gene (Fu *et al*., 2012). The tissue-specific expression of *ZmWRKY86* at different developmental stages of maize was assessed (by qRT-PCR) and it was observed that the maximum expression of this gene was in stems, followed by roots and leaves, while minor expression was observed in the embryo. It also studied the expression patterns of the *ZmWRKY86* gene under different abiotic stresses, including drought stress and salt stress, and it was observed that under NaCl salt stress conditions, the expression of the *ZmWRKY86* gene involved the regulation of salt stress-responsive genes by controlling the expression of stress-related genes, as well as the plant survival rate,compared with the untreated control sample (Fang *et al*.,2021).

Table (1): Gene primers *ZmWRKY8* and *PMP3* and housekeeping genes are used in the RT-PCR technology.

Primer	Sequence
PMP3-F	GCCGCAGGTGGAGGAAGG
PMP3-R	GGTAGCCGAGGATGGTGAGCA
ZmWRKY8-F	GTGTCAGCTGTTCAGGATGT
ZmWRKY8-R	AAGTAGGACCTCGGGTACTTAG
Housekeeping gene-F	GAAGAGCCGCAAAGTTATGG
Housekeeping gene-R	ATGGTAGAAGTGGACGCACC

Table (2): Components of the final reaction volume for the RT-PCR reaction

T	Components	Volume
1	cDNA	**5 µl**
2	F_primer	**1 µl**
3	R_primer	**1 µl**
4	Greenq PCR super mix	**10 µl**
5	D.W	**3 µl**
	Total volume 20 µl	

Table (3): Steps of the program in the RT-PCR reaction.

No.	Stage	Temperature	Time	Number of Cycles
1.	Initial denaturation	95	5 min.	**1**
2.	Denaturation	95	1 min.	**35**
3.	Annealing	58	1 min.	
4.	Extension	72	1 min.	
5.	Final extension	72	5 min.	**1**
6.	Stop reaction	4	5 min	**1**

Table (4): germination percentage at salt stress.

	Concentration of NaCl salt / mmol	Germination percentage %
1	Control	90
2	25	80
3	50	70
4	75	50
5	100	30

Table (5): Analysis of Gene expression levels for (*PMP3* gene) that correlated with salt tolerance

PMP3 gene						
Conc. of salt (mmol)	**CT target gene**	**CT housekeeping gene**	**Δ CT target gene**	**Δ CT control**	**Δ Δ CT**	**Gene Expression folding**
0	26.723675	27.602985	------	-0.87	0	1
25	26.053412	26.680106	-0.62	-0.87	0.25	0.84
50	26.084408	27.428845	-1.34	-0.87	-0.47	1.38
75	26.72514	28.499326	-1.77	-0.87	-0.9	1.86
100	27.945268	27.272087	0.67	-0.87	1.54	0.34

Table (6): Analysis of Gene expression levels for (*ZmWRKY86* gene) that correlated with salt tolerance

ZmWRKY86 gene						
Conc. of salt (mmol)	**CT target gene**	**CT housekeeping gene**	**Δ CT target gene**	**Δ CT control**	**Δ Δ CT**	**Gene Expression folding**
0	34.185237	27.602985	------	6.58	0	1
25	33.32637	26.680106	6.64	6.58	0.06	0.95
50	35.395667	27.428845	7.96	6.58	1.38	0.38
75	34.369948	28.499326	5.87	6.58	-0.71	1.63
100	32.185237	27.272087	4.91	6.58	-1.67	3.18

Conclusion

In this research, the results indicated that the germination percentage decreased with the increased salt concentration and the gene expression of the *ZmWRKY8* and *PMP3* genes that are responsible for tolerance to salinity stress was measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR) from young leaves of maize seedlings, and it was observed that their gene expression increased with increasing sodium chloride salt concentration especially *ZmWRKY8* gene.

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Contributions of authors

H.H.T. carried out the experiment in the field and collected the data; wrote the manuscript

N.I.K. wrote the manuscript; data analysis

A.A.M. constructed the idea and hypothesis for research; planned the methodology

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Conflicts of interest

The authors declare that they have no conflict of interests.

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تحديد التعبير للجينات *ZmWRKY86* و*PMP3* المسؤولان عن تحمل الاجهاد الملحي لنبات الذرة الصفراء

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المستخلص: قامت الدراسة الحالية بقياس تعبير بعض الجينات المسؤولة عن تحمل نبات الذرة الصفراء (*Zea mays* L.) لظروف الإجهاد الملحي عند نموه في تربة تحتوي على (25، 50، 75، 100 ملي مول) من كلوريد الصوديوم (NaCl). أشارت النتائج بعد 30 يوما إلى أن نسبة الإنبات انخفضت مع زيادة تركيز كلوريد الصوديوم. تم عزل الحمض النووي RNAمن الأوراق الصغيرة لشتلات الذرة كقالب لتصنيع قطعة من الحمض النووي التكميلي (cDNA)، وتم قياس التعبير الجيني بواسطة تفاعل البوليمير المتسلسل للنسخ العكسي (RT–PCR) لكل من جينات *PMP3* و*ZmWRKY86* المسؤولان عن تحمل إجهاد الملوحة، لوحظ ارتفاع مستوى التعبير للجين *PMP3* مع زيادة تركيز ملح كلوريد الصوديوم ووصل إلى 1، 2.82، 4.59 و6.49 عند التراكيز 0.0، 25، 50 و75 ملي مول على التوالي، ولكنه انخفض مع الزيادة إلى 100 ملي مول. لوحظ زيادة تعبير الجين الثاني *ZmWRKY86* مع زيادة تركيز ملح كلوريد الصوديوم ووصل إلى اعلى مستوى قدره 3.03 عند 100 ملي مول من كلوريد الصوديوم.

الكلمات المفتاحية: نبات الذرة، تحمل الملوحة، جين تحمل الملوحة، التعبير الجيني.