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ORIGINAL ARTICLE

Expression Analysis of Salinity-Related Genes in Bread Wheat (*Triticum aestivum* L.) Varieties and Lines

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ABSTRACT
Wheat (Triticum aestivum L.) is the main crop for food consumption in Uzbekistan. Selecting wheat genotypes that are suitable for specific conditions is challenging because the biological and beneficial properties of wheat are determined by gene combinations and vary according to environmental factors. In the climatic conditions of Uzbekistan, introducing stable varieties of wheat for yield and quality is considered economically necessary. The aim of this research is to identify the salt tolerance capacity of varieties grown in Uzbekistan and lines belonging to the ICARDA gene bank, as well as to reveal the expression levels of salinity-related genes (SOS1, SOS2, SOS3, SOS4, LEA1, WRKY1, P5CS1, and Actin as a control gene). It was found that the expression levels of these genes vary significantly in salt-tolerant E'zoz and salt-sensitive FAWWON-43 samples.
Keywords: *Gene markers, NaCl, (qRT-PCR) and Ta_LEA1*

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is considered a strategic crop for food in Uzbekistan. However, today, abiotic stress factors such as drought, cold, and soil salinity are among the most significant contributors to reduced yields.

High soil salinity is a major abiotic stress in global crop production [1]. Increasing salt tolerance in the two main crops—wheat and rice—is an important goal, as the demand for these crops is rising daily. The expansion of urban areas is leading to a reduction in the available land for agriculture, which necessitates increasing productivity to maintain global food supply. Additionally, land reclamation or the rise in water levels due to irrigation can lead to increased salinity of the land [2].

Salinity at certain levels is a primary limiting factor in food production, and many drought-prone regions around the world experience both natural and complex salinity [3]. This study was conducted to investigate the salt tolerance mechanisms of wheat (*Triticum aestivum* L.) under saline conditions. For this purpose, 14-day-old seedlings of some local salt-tolerant wheat varieties, specifically E'zoz, and the salt-sensitive line FAWWON-43, were exposed to various concentrations of NaCl (0, 100, and 200 mM) for 48 hours. Subsequently, an experiment was conducted to assess the expression of salinity-related genes (SOS1, SOS2, SOS3, SOS4, P5CS1, LEA1, and WRKY1). The aim of this experiment is to elucidate the mechanisms by which salt tolerance affects plants in both salt-tolerant and salt-sensitive varieties.

MATERIAL AND METHODS

Plant materials. The salt-tolerant variety E'zoz of soft wheat (*T. aestivum* L.), developed at the Uzbekistan Research Institute of Genetics and Experimental Plant Biology, and the salt-sensitive line FAWWON-43 from ICARDA were used in this study. The seeds were sterilized in 70% ethanol for 4 minutes and then washed with sterile water. The sterilized wheat seeds germinated in a growth chamber at 25 °C.

Fourteen-day-old seedlings were transferred to petri dishes containing 100 and 200 mM NaCl solutions and grown under light for 48 hours. Unprocessed wheat seedlings were considered as the control. Leaf samples were frozen in liquid nitrogen and stored at -70 °C until RNA extraction.

RNA isolation and cDNA synthesis.

RNA Extraction: Wheat samples from salt-tolerant and salt-sensitive varieties were grown at NaCl concentrations of 0, 100, and 200 mM. Leaf samples from the plants grown under these conditions were collected and stored at -80ºC. Total RNA was extracted using the RNeasy PowerPlant Kit (cat. no. ID: 13500-50). RNA isolation was performed on ice according to the kit protocol (https://www.protocols.io/view /qiagen-rneasy-plant-rna-extraction-protocol-modifi-bwjepcje).

cDNA Preparation: cDNA synthesis was carried out using the SuperScript™ III Reverse Transcriptase, 2,000 units cDNA synthesis kit (USA). The cDNA synthesis reaction was incubated in a programmed PCR amplifier (BioRad T100TM Thermal Cycler, Singapore) for one cycle at 40ºC for 40 minutes and then at 95ºC for 10 minutes.

To determine the expression of the relevant salinity-related genes in wheat samples, PCR analysis was performed using specific primers with the "2X SYBR® Green PCR Master Mix." The reaction was set up as follows: 10 µL of 2X SYBR® Green PCR Master Mix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 2.0 µL of cDNA, and 7.0 µL of water, for a total volume of 20 µL for each RT-PCR reaction. Primers for real-time PCR (RT-PCR) analysis of these genes were designed using the Integrated DNA Technologies (IDT, USA) online platform (https://eu.idtdna.com/scitools/Applications/RealTimePCR/default.aspx).

qRT-PCR reactions were conducted with the X 960 Real-time PCR System Model No: X960B-5 (Heal Force International Trading (Shanghai) Co., Ltd, China) with the following cycling parameters: 3 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 55°C, and 40 seconds at 72°C for 40 cycles. The Actin gene was used as a control gene for analyzing the expression of salinity-related genes. The relative expression level of mRNA (Relative gene expression) was calculated based on the formula by K.J. Livak (2001).

RESULTS

To determine the expression of salinity-related genes SOS1, SOS2, SOS3, SOS4, LEA1, WRKY1, P5CS1, and the control gene Actin in *Triticum aestivum* L. under different concentrations of NaCl (0, 100, and 200 mM), primers for these genes were designed for real-time PCR (RT-PCR) analysis targeting mRNA using the Integrated DNA Technologies (IDT, USA) online platform and ordered accordingly (Table 1).

Table 1. Primers specific to the genes used for RT-PCR analysis of wheat.

№	Primer name	Sequence3'-5'
1	SOS1_F	GTTGTCGGTGAGGTCGGAGGG
	SOS1_R	TCATCTTCTCCTACCGCCCTGC
2	SOS2_F	CTGCCCAAGGAAATGTTCAG
	SOS2_R	ATCCTATTCCTGTACACCACCAGT
3	SOS3_F	GAAGAACATGACTCTCCCATACCT
	SOS3_R	ACATGATGTTGTATGCCTGAGACT
4	SOS4_F	TGAGATACCCAAGATACCTGCATA
	SOS4_R	TGATCTCATCCTGGCTTTGTATTA
5	WRKY1_F	GCATCCTAGGGGTTACTACAAGTG
	WRKY1_R	TCTTTCTCTAGAAAACGGAGGCTA
6	P5CS1_F	GATCTTGTTATTCCAAGAGGCAGT
	P5CS1_R	AAGCAGTGTTTCCATAGCATTACA
7	LEA1_F	CAAGGCCGGCATCGATAA
	LEA1_R	TCGTCGGAGTGGACCTT
8	Actin_F	CTTGTATGCCAGCGGTCGAACA
	Actin_R	CTCATAATCAAGGGCCACGTA

During the study, electrophoresis was performed using 1.5% Agarose D1 High EEO gel at 125 V for 70 minutes (CS-300V). The visual image of the gel was captured using the GelDog imaging system (GelDog Go USA) (Figure 1).

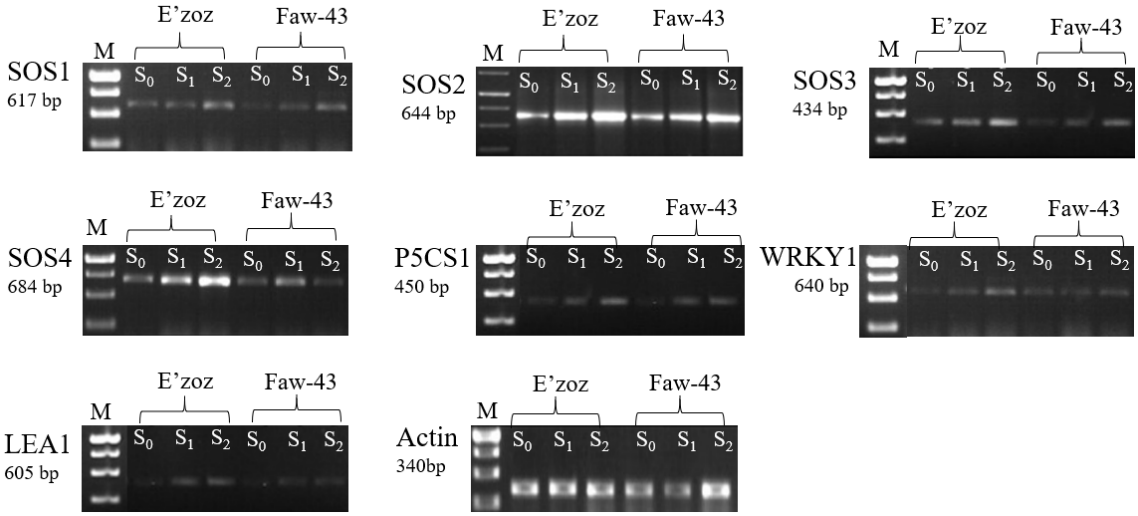

Figure 1. Electropherogram of salinity-related genes and the Actin gene in 1.5% agarose gel. (S_0 - Control 0 mM NaCl, S_1 - 100 mM NaCl solution, S_2 - 200 mM NaCl solution)

In the salt-tolerant variety E'zoz, we can observe that the expression of salinity-related genes increases with the concentration of salt. In the salt-sensitive sample Faw-43, it was found that the expression of the genes SOS1, SOS2, and SOS3 also increased with higher concentrations. However, the SOS4 and LEA1 genes initially had low expression levels in the S0 control (normal conditions). Under the saline stress of 100 and 200 mM NaCl, their expression was found to be very low. The Actin gene was used as a control gene for evaluating the expression of SOS1, SOS2, SOS3, SOS4, and LEA1. The expression of the Actin gene was not dependent on salt stress, which confirmed that it was expressed at similar levels across different concentrations in the selected samples.

To assess the actual relative quantities of the genes, qRT-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR reactions were set up in a total volume of 25 µL as follows: 10 minutes at 95°C, then 15 seconds at 95°C, and 45 seconds at 65°C for 40 cycles. Each 25 µL reaction mixture consisted of 12.5 µL SYBR Green Master Mix (2×), 0.35 µL (140 nM) of each primer (10 µM), 6 µL of 1:15 diluted cDNA, and 5.8 µL of sterile water. The 2× SYBR Green PCR master mix contained No AmpErase UNG, AmpliTaq Gold DNA polymerase, dUTP with deoxynucleotide triphosphates, and SYBR Green magnesium chloride reaction buffers (Applied Biosystems, Waltham, MA, USA).

To prevent errors in the reactions, all assays were performed with three biological replicates and three technical repeats for each biological sample. All primers were developed using the Integrated DNA Technologies Inc. (Coralville, IA, USA) Primer3 and IDT OligoAnalyzer Tool.

The actual relative quantities of the genes were assessed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the available software. Graphs were created using GraphPad Prism version 8.0.1 (www.graphpad.com; GraphPad Software, San Diego, California, USA).

According to the analysis results, the salinity-related Ta_SOS1 gene was found to be expressed at similar levels in the salt-tolerant variety E'zoz and the salt-sensitive line Faw-43 when grown in optimal conditions, with fold change (FC=2^-ΔΔCt) values of 1.18 and 1.15, respectively, in a 100 mM NaCl solution. In a 200 mM NaCl solution, the expression of the Ta_SOS1 gene was measured at 1.645 in the salt-tolerant E'zoz and 1.32 in the salt-sensitive Faw-43. There was no significant difference in the expression levels of the Ta_SOS1 gene in both wheat samples, indicating that the importance of this gene in salt tolerance for the selected wheat varieties is relatively low compared to other genes.

Further analysis revealed that the Ta_SOS2 gene was also expressed in a similar manner in the salinity-related E'zoz and salt-sensitive Faw-43 varieties. It was determined that the expression level of the Ta_SOS2 gene increased with the rise in NaCl concentration in the optimal growth environment. In the E'zoz variety, the fold change (FC=2^-ΔΔCt) was 1.596 at 100 mM NaCl concentration and increased to 1.988 at 200 mM. This indicates that the salt-tolerant E'zoz variety expresses the Ta_SOS2 gene at a high

level. In contrast, the expression levels of the Ta_SOS2 gene in the Faw-43 line were significantly lower, with values of 1.235 and 1.645 at 100 mM and 200 mM NaCl concentrations, respectively.

Thus, the expression level of the Ta_SOS2 gene is significantly higher in the E'zoz variety, indicating its greater importance in salt tolerance compared to the Faw-43 line. The increase in NaCl concentration correlates with an increase in the expression of the Ta_SOS2 gene, which suggests that this gene may play an important role in the process of salinity tolerance. Additionally, the observed differences in the expression of the Ta_SOS2 gene among the studied varieties indicate that it is strongly expressed in the salt-tolerant E'zoz variety while showing relatively low expression in the salt-sensitive Faw-43 line. This suggests that the Ta_SOS2 gene has an important role in salt-tolerant wheat varieties.

Analysis of the Ta_SOS3 gene showed that the expression level of this gene also changed with varying NaCl concentrations in the E'zoz and Faw-43 varieties, similar to other genes. In the E'zoz variety, the expression level in the control condition had a fold change (FC=2^-ΔΔCt) value of 1.04645. When the NaCl concentration was increased to 100 mM, this value rose to 1.18734, and at 200 mM, it reached 1.38435. These results indicate that the expression level of the Ta_SOS3 gene increased significantly with the rising NaCl concentration in the E'zoz variety, suggesting that the Ta_SOS3 gene plays an important role in supporting the salinity tolerance mechanisms of this variety.

In the Faw-43 line, the expression level in the control condition was also similar to that of the E'zoz variety, with a fold change (FC=2^-ΔΔCt) value of 1.04353. At 100 mM NaCl concentration, this value was 1.13474, and at 200 mM, it was 1.24435. While the expression of the Ta_SOS3 gene in the Faw-43 variety also increased with the NaCl concentration, this increase was less pronounced compared to the E'zoz variety.

Overall, the differences in the expression levels of the Ta_SOS3 gene help elucidate its role in the process of salinity tolerance. The higher expression level in the E'zoz variety indicates that the Ta_SOS3 gene plays a crucial role in developing salinity tolerance mechanisms, while the Faw-43 line shows less effectiveness in this process.

Our analysis of the Ta_LEA1 gene indicates that there are significant differences in the expression levels of this gene with varying NaCl concentrations in both E'zoz and Faw-43 varieties. Specifically, in the E'zoz variety, the expression level in the control condition had a fold change (FC=2^-ΔΔCt) value of 1.00231. When the NaCl concentration was increased to 100 mM, this value rose to 1.78554, and at 200 mM, it reached 2.72289. These results demonstrate that the increase in NaCl concentration plays a very important role in the expression of the Ta_LEA1 gene in the E'zoz variety, indicating its high significance in supporting salinity tolerance mechanisms.

In the Faw-43 line, the expression level in the control condition was 1.00086. However, at 100 mM NaCl concentration, this value dropped to 0.45655, and at 200 mM, it decreased further to 0.08945. These results show that the increase in NaCl concentration significantly reduces the expression level of the Ta_LEA1 gene in the Faw-43 variety, indicating its sensitivity to salinity.

Overall, the differences in the expression levels of the Ta_LEA1 gene help us understand its role in the salinity tolerance process of wheat varieties. The high expression of the Ta_LEA1 gene in the E'zoz variety indicates its significant importance in developing salinity tolerance mechanisms. Thus, the sensitivity of the Ta_LEA1 gene to NaCl concentrations may create new opportunities for enhancing the salinity tolerance of wheat. These analyses may also allow for the identification of nucleotide sequences in the Ta_LEA1 gene among tolerant and sensitive wheat varieties, revealing the mechanisms of salinity tolerance.

According to the research results, the expression levels of the salinity-related genes Ta_WRKY1 and Ta_P5CS1 in both salt-tolerant and salt-sensitive wheat samples increased with rising NaCl concentrations. Specifically, the expression of the Ta_WRKY1 gene increased in both varieties. In the E'zoz variety, the expression level in a 100 mM NaCl solution was found to be 1.49657 compared to the control, and in a 200 mM NaCl solution, it increased to 1.87644. In the Faw-43 variety, the expression levels were 1.33455 at 100 mM NaCl and 1.74534 at 200 mM NaCl (Figure 2).

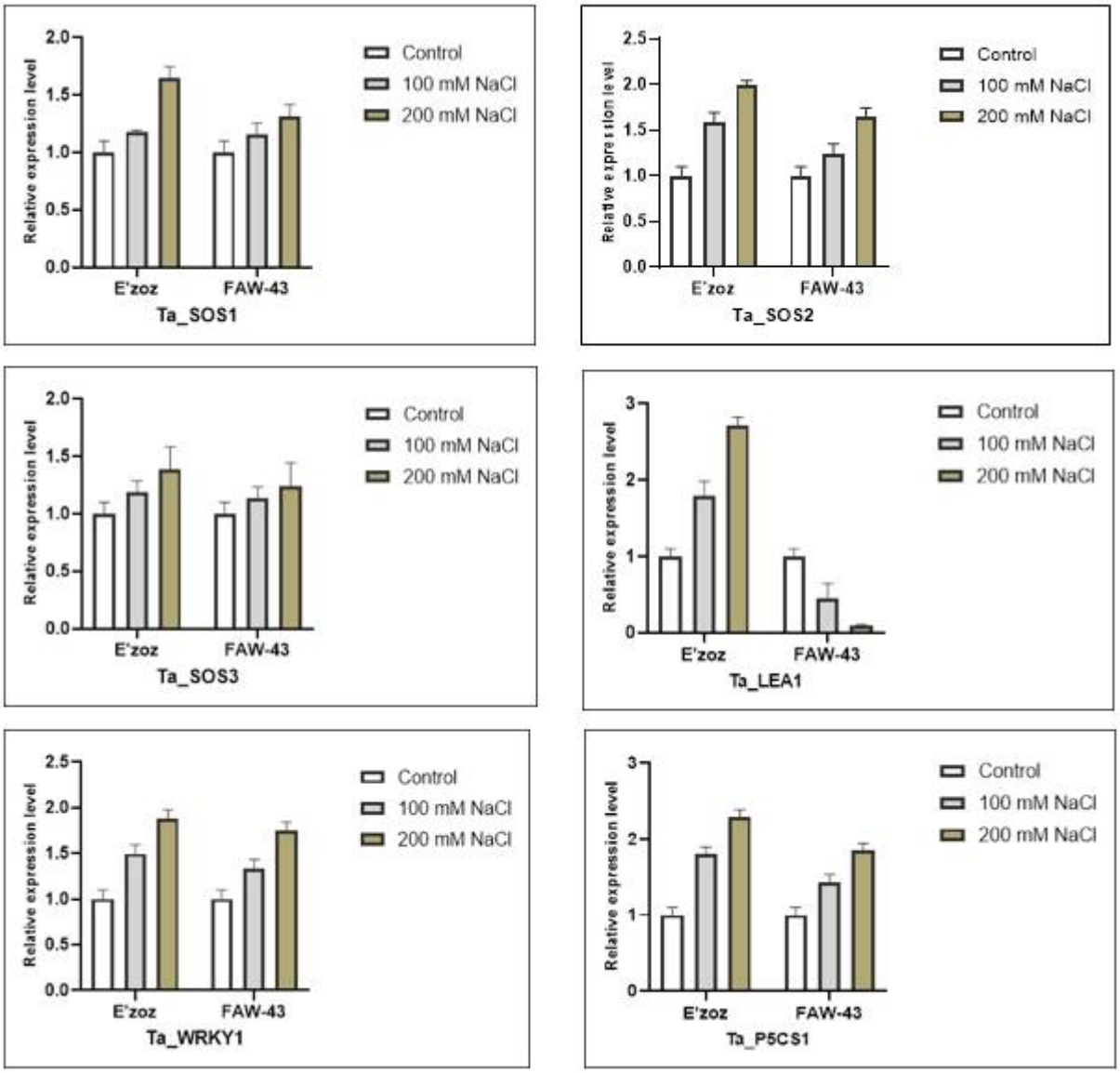

Figure 2. Overall expression levels of the genes SOS1, SOS2, SOS3, LEA1, WRKY1, and P5CS1 in salt-tolerant E'zoz and salt-sensitive Faw-43 wheat varieties grown under various concentrations of NaCl (0, 100, and 200 mM).

Our analysis results further indicate that the Ta_SOS4 gene regulates the transport of Na+ and K+ ions. Due to its activity, salt and water homeostasis are maintained, protecting cells from salinity stress. The SOS4 gene works in conjunction with other members of the SOS gene family and also regulates the expression of other genes in response to stress.

When analyzing the expression of the Ta_SOS4 gene, a difference in gene expression levels was observed between the salt-tolerant E'zoz and salt-sensitive Faw-43 lines. According to the results, the expression level of the Ta_SOS4 gene in the salt-tolerant E'zoz variety in a 100 mM NaCl solution was 0.62935, indicating that the gene expression was low compared to the control condition. However, in a 200 mM NaCl solution, this value increased to 2.35507, showing an increase in gene expression levels in a saline environment.

In samples of the E'zoz variety exposed to a 100 mM NaCl solution, the expression of the SOS4 gene decreased to 0.25 log2 compared to the control (1 log2). In samples from the E'zoz variety exposed to a 200 mM NaCl solution, the expression of the SOS4 gene increased twofold to 2 log2 compared to the control.

In the salt-sensitive Faw-43 line, the expression of the SOS4 gene in samples exposed to a 100 mM NaCl solution was very low, at 0.0078 log2 compared to the control (1 log2). In samples from the Faw-43 line exposed to a 200 mM NaCl solution, the expression was also low, at 0.0156 log2 compared to the control (Figure 3).

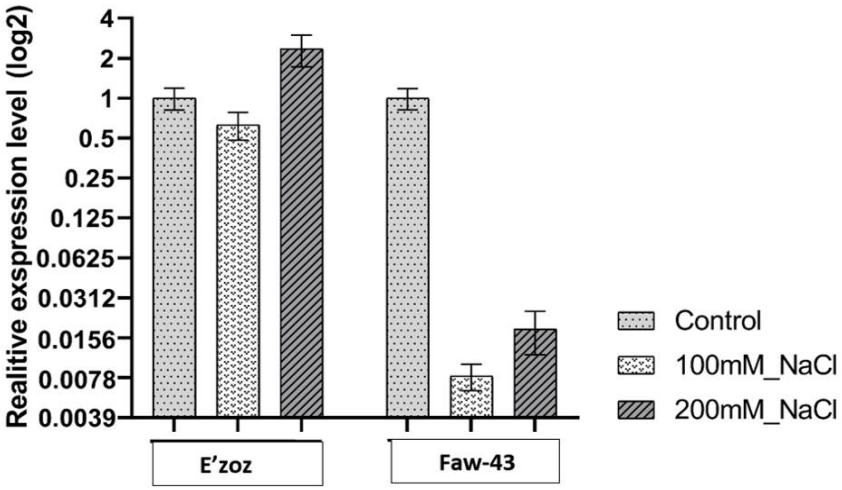

Figure 3. Overall expression levels of the SOS4 gene in salt-tolerant E'zoz and salt-sensitive Faw-43 wheat varieties grown under various concentrations of NaCl (0, 100, and 200 mM).

Overall, the expression of the Ta_SOS4 gene increased with rising NaCl concentrations in the E'zoz variety, while in the Faw-43 line, the expression level decreased with increasing NaCl concentration.

DISCUSSION

The findings of this study highlight the differential expression of salinity-related genes in the salt-tolerant E'zoz variety and the salt-sensitive Faw-43 line of wheat (*Triticum aestivum* L.) in response to varying NaCl concentrations. The expression levels of genes such as Ta_SOS1, Ta_SOS2, Ta_SOS3, Ta_SOS4, P5CS1, WRKY1, and LEA1 were significantly affected by salinity stress, confirming their roles in the plant's adaptation mechanisms.

In the E'zoz variety, the expression of the Ta_SOS4 gene increased with higher NaCl concentrations, which aligns with previous studies indicating that this gene regulates Na+ and K+ ion transport, thus maintaining salt and water homeostasis. This suggests that Ta_SOS4 plays a crucial role in protecting cells from salinity stress, as noted by [Ganeshan, S., 2008]. The increase in expression in the E'zoz variety compared to the decrease observed in the Faw-43 line supports the notion that E'zoz has developed effective mechanisms to cope with salinity.

Additionally, the Ta_LEA1 gene exhibited the highest expression levels in the E'zoz variety, reinforcing its importance in salinity tolerance as highlighted by [Chen, 2015]. This gene is known for its role in protecting cellular structures during abiotic stresses, including salinity. Conversely, the downregulation of Ta_LEA1 expression in the Faw-43 line under salt stress indicates its vulnerability to salinity, which is consistent with findings from [Ling, 2016].

The significant upregulation of the WRKY and P5CS1 genes in the E'zoz variety further supports the evidence that these genes are integral to the salinity tolerance mechanisms in wheat. Previous research has shown that WRKY transcription factors are involved in stress responses and the regulation of downstream genes related to osmoregulation [Niu C, 2012].

In summary, the results of this study underscore the critical roles of the examined genes in enhancing salinity tolerance in E'zoz wheat, which could provide valuable insights for future breeding programs aimed at developing salt-resistant crop varieties. The differential expression patterns observed between the salt-tolerant and salt-sensitive lines could inform selection strategies to improve crop resilience to saline environments.

CONCLUSION

In conclusion, our results show that the genes WRKY1, SOS1, SOS2, SOS3, SOS4, P5CS1, and LEA1 are expressed differently in E'zoz (salt-tolerant) and FAWWON-43 (salt-sensitive) wheat varieties with increasing NaCl concentrations. In the E'zoz variety, gene expression significantly increased with rising NaCl concentrations, indicating that these genes play an important role in salinity tolerance.

The salinity-related Ta_LEA1 gene achieved the highest expression level in the E'zoz variety, confirming its salt tolerance. In contrast, the increase in NaCl concentration in the Faw-43 line led to a decrease in gene expression, indicating its sensitivity to salinity. Overall, this study emphasizes the important role of these genes in enhancing the salinity tolerance of the E'zoz variety and may serve as a basis for future selection and agronomic strategies.

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