RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
54 (4) 780-788, 2022
http://doi.org/10.54910/sabrao2022.54.4.9
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

TAOPR1 SALT TOLERANCE GENE EXPRESSION AND PHYSIOLOGICAL TRAITS IN WHEAT

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SUMMARY

Salinity is an abiotic stress factor and a major challenge that has significant negative effects on wheat production. It is also a source of concern for plant breeders leading them to reach reliable screening criteria for salt tolerance in wheat genotypes. The physiological analysis showed that the three salt-tolerant wheat genotypes viz., Dijla, 2H, and 3H showed the highest rate for the physiological traits i.e., chlorophyll content (38.9, 39.5, and 42.1, respectively), carbohydrates (600.14, 590.6, 560.8: 2H, 3H, and Dijla, respectively), proline acid (24.30, 23.14, and 21.87: Dijla, 3H, and 2H, respectively) under salt stress conditions, except protein percentage (3.8% and 3.3%: Rabia and Ibaa99, respectively) and K^+/Na^+ ratio (6.3 and 5.9: 2H and Dijla, respectively). The salt-tolerant wheat genotypes 2H, Dijla, and 3H enunciated an increased rate of expression of salt-related genes (TaOPR1 gene and *β*-actin gene) with values of 6.498, 4.0, and 3.768, respectively compared to two other salinity-sensitive cultivars i.e., Ibaa99 and Rabia under salt stress conditions. The salinity-sensitive cultivars i.e., Ibaa99 and Rabia showed no gene expression and significant difference with the control treatment after being treated with salinity stress conditions.

Keywords: wheat (*Triticum aestivum* L.), gene expression, TaOPR1 salt tolerance gene, salt-tolerant genotypes, chlorophyll, protein, carbohydrates, proline acid, K^+/ Na^+

Key findings: Under saline stress conditions, the genotypes of Dijla, 2H, and 3H showed the highest content of chlorophyll, protein (%), carbohydrate, and proline acid, except for K^+/Na^+ ratio, and excelled other wheat genotypes in gene expression (6.498, 4.0, and 3.768), respectively at the salt level of 16 dS/m. However, the salinity-sensitive wheat genotypes Ibaa99 and Rabia provided relatively low values for the expression of TaOPR1 gene expression associated with salt tolerance.

Communicating Editor: Prof. Naqib Ullah Khan

Manuscript received: September 13, 2022; Accepted: Octoaber 23, 2022.

INTRODUCTION

Wheat is one of the most important and ancient grain crops that have been domesticated by humans. Wheat accounts for 20–25% of the total calories that humans require on a daily basis for their diet, making it a vital crop that is cultivated and used as a staple meal in many nations across the world (Gill, 2004). In developing countries, the

To cite this manuscript: Majeed DM, Ismail EN, Al-Burki FR, Abed AS, Al-Jibouri AMJ (2022). TAOPR1 salt tolerance gene expression and physiological traits in wheat. *SABRAO J. Breed. Genet.* 54 (4) 780-788. http://doi.org/10.54910/sabrao2022.54.4.9

agricultural sector faces several challenges, including abiotic stress, which causes significant losses through a negative impact on the growth and productivity of crops, including wheat (Dwivedi *et al*., 2018; Gaballah *et al*., 2021; Farhood *et al*., 2022). Salinity is the second most important abiotic stress after drought, which significantly affects wheat production, and it showed limited tolerance to the salinity stress (Al-Burki *et al*., 2019). Salinity stress leads to negative effects on the transport chain of elements and cell enlargement and division in the growth areas of the plants, as well as reducing the growth, dry mass, leaf size, and eventually decrease the grain production (EL-Sabagh *et al*., 2021). In wheat, the salinity tolerance mechanism results from a complex interaction between environmental and genetic factors, including reducing Na^+ ion uptake by the roots, restricting it within the vacuoles, and excluding it from the older leaves (Ismail and Horie, 2017).

Physiological parameters in wheat are very important because of their relation to the growth and development processes. Past findings indicated that the chlorophyll content affects the qualitative characteristics, and in wheat flour, the protein content showed a very close relationship with the chlorophyll content (Szabó, 2014). The regulation of sodium uptake and transport in plants under the influence of salt stress on a large scale to maintain high levels of K^+/Na^+ ratio in the tissues and cytosol has become an essential feature of salt stress tolerance (Shabala and Pottosin, 2014). Since K^+ is involved in a lot of physiological functions in plants, the high Na^+ concentration inhibits its uptake competitively, and K^+ deficiency becomes severe under salinity, resulting in poor growth, and K^+ deficiency is the major cause of sensitivity to salinity stress (Véry and Sentenac, 2003), as well as affecting the carbohydrate and proline acid concentrations.

The negative effects of salt stress on plant growth and water content may be due to a metabolic defect in plant cells (Al-Khafaji and Al-Burki, 2021). Applied salinity levels increased the activity of Na^+ and Cl^- contents, ascorbate, and H_2O_2, in wheat plant leaves compared to the control treatment (Fercha *et al*., 2011). In addition, the increase in proline, protein, carbohydrates, and ascorbate was greater under 12 ds/m NaCl compared to the control treatments (Rady *et al*., 2019). Munns and Termaat (1986) findings indicated that plant tissues vary greatly in carbohydrate content and their distribution among plant organs which may be degraded by salinity because various tissues respond differently to salinity. It is believed that the accumulation of carbohydrates in plant leaves in the presence of salinity occurs mainly as a result of reduced exports (Munns *et al*., 1982). The content of carbohydrates and soluble proline have also been significantly increased at the four salinity levels (50, 100, 150, and 200 mM) in different parts of the two wheat cultivars (Shaddad *et al*., 2013).

Saline stress is regulated by OPRI genes, and the analysis of cis-acting in promoters showed that the OPRs gene performs various functions in the wheat plants related to biotic and abiotic stresses, growth, hormones, and development (Mou *et al*., 2019). The TaOPR1 gene expression responds to salt stress and its over-expression in wheat plants improves salt tolerance, and this effect is operated by the regulation of ABA and ROS signaling pathways (Dong *et al*., 2013). RT-qPCR analysis indicated that TaOPR1 mRNA levels were significantly increased in cultivar R3 compared to genotype JN177 after salt treatment (Ejaz *et al*., 2020). To understand the mechanism of gene expression, the values of the gene expression mostly depend on the environmental stress degrees. Gene expression increases with salinity enhancement because gene expression is a type of interaction between genetic and environmental factors. Therefore, it must be investigated and the gene associated with a salinity tolerance must be accompanied by an estimation of gene expression and determining the level of salinity that motivates it to express (Al-Mishhadani, 2012). Based on the above mentioned facts, this study aimed to estimate the salt tolerance gene TaOPR1 expression and analysis of some physiological parameters in selected genotypes of wheat (*Triticum aestivum* L.).

MATERIALS AND METHODS

Plant material and

The present experiment was carried out from 2021 to 2022 in a randomized complete block design with three replicates in the Laboratory of the Biotechnology Research Center, Al-Nahrain University, Baghdad. Three salinity tolerant selected wheat genotypes (2H, 3H, and Dijla) resulted from a plant breeding program and two Iraqi local cultivars Ibaa99 and Rabia (control) were used to determine the saline-resistant gene (TaOPR1) and its expression under saline conditions. The seeds

of the wheat cultivars (five seeds in each pot) were sown in the saline soil prepared at three levels : 2, 8 and 16 ds/m (were created in accordance with the approach described by Gupta *et al*. (2012) by including the appropriate amounts of NaCl, $CaCl_2$, and Na_2SO_4 salts in the soil at the appropriate concentration), under greenhouse conditions, by using five seeds per pot and with three replications per treatment, and the plants grew under saline conditions for 50 days from the date of sowing. Later, the leaf samples were collected for RNA extraction.

Chlorophyll Content (SPAD)

Five leaves from each cultivar were randomly taken and measured by SPAD unit (Singh and Ali, 2020) by taking the measurements from four different regions in leaf by chlorophyll meter (SPAD-502, Minolta company-Japan).

Protein (%)

The grains were dried at 70 °C for 72 h and then ground, 0.2 g dry sample was taken, the sample was digested in solution (Perchloric acid 50% + Sulfuric acid 98%) percent (1:1). Digest sample entered in Micro Kjeldahl Device (A.O.S.A., 2000), and protein measured according to the following equation (Bruckner and Morey, 1988):

$$Protein\ (\%) = N(\%) \times 5.83$$

Sodium and Potassium Content (mg g^{-1} sample)

The 0.1 g from the dry shoot was digested in 2.5 ml (H_2SO_4 98%) with 1.5 ml (Perchloric acid 50%), The samples were heated with shaking, and the solution color changed to a colorless solution, then the solution was left in room temperature and K^+ and Na^+ ions were estimated using a flame photometer (Hard, 1946).

*Potassium / Sodium ratio (K^+ / Na^+) : calculated from the equation:

Percent Potassium / Sodium = K^+ content / Na^+ content

Concentration of carbohydrates (mg g^{-1})

The concentration of carbohydrates was estimated according to Herbert *et al*. (1971), using a spectrophotometer at a wavelength of 490 nm.

Concentration of proline acid (µg g^{-1})

The concentration of proline acid was estimated according to Ábrahám *et al.* (2010), using a spectrophotometer at a wavelength of 520 nm.

RNA Isolation and cDNA Synthesis

Total RNA was isolated by using a Geneaid RNA purification mini kit (Taiwan) following the manufacturer's instructions. By using RNase-free DNase-I (Biobasic, Canada), the isolated RNA was processed at 37 °C for 20 min, and DNase-I was inactivated at 65°C for 10 min. RNA integrity was verified after isolation by gel electrophoresis on a 1.5% agarose containing 0.5% (v/v) ethidium bromide. RT System (Pioneer, Korea) with oligo-dT15 was used to synthesize from 500 ng of total RNA, and the reaction solution was used as a template for Quantitative Real- Time PCR (RT-PCR) (Ismail, 2015).

The target gene (TaOPR1) and wheat housekeeping reference gene (β-actin) cDNA were amplified using specific primers (Table 1), the primers in this experiment were designed using the NCBI website. PCR reaction was initiated with a hot start system by using the template of cDNA (Labnet Thermo cycler-USA), and it was programmed using standard protocol: 95 °C (5 min) and 40 cycles at 95 °C (60s), 58 °C (45s), and 72 °C (60s).

Table 1. The primers with their sequences used in the study.

Genes	Foreword	Reverse	Product length (bp)	References
B-actin	TGGCACCCGAGGAGCACCCTG	GCGACGTACATGGCAGGAACA	100	AF326781.1 (Guang and Liang, 2011)
TaOPR1	GCGGCTATTCCTGGCAAAC	GACGGGATCGGAGATGTAGAAC	110	NCBI (Primer design)

Gene expression

Relative Real-Time (SYBR Green Dye) was approved to analyze TaOPR1 gene expression using (Exicycler real-time PCR - Bioneer, Korea). Following the manufacturer's protocol, and to confirm amplification specificity, the thermal cycling was carried out with melting curve analysis at 60-95°C, and quantization of relative expression was determined by the $2^{-\Delta\Delta ct}$ method, and each sample was run in triplicate (Livak and Schmittgen, 2001).

$$\text{Relative Gene Expression} = 2^{-\left(CT_{Trget\ gene} - CT_{\beta-actin}\right)_{Test} - \left(CT_{Target\ gene} - CT_{\beta-actin}\right)_{Control}}$$

The cycle threshold (CT) of a target gene is denoted by the variable CT target, while the cycle threshold (β-actin) of the reference gene is denoted by CT β-actin. It is the difference between the cycle threshold of the gene being targeted and the cycle threshold of the gene serving as a reference for the samples being analyzed. The term 'control' refers to the differences in cycle thresholds observed between the target gene and the reference gene in samples used for quality control.

RESULTS

Salinity effects on chlorophyll content (SPAD)

The results revealed that wheat genotypes significantly differed in the chlorophyll content under the conditions of saline and non-saline soil (Table 2). By increasing salty levels from 2 to 16 ds/m, the chlorophyll content increased significantly in the tolerant genotypes Dijla, 2H, and 3H which reached 38.9, 39.5, and 42.1 SPAD, compared to the control treatments (34.2, 30.8, and 33.5 SPAD) respectively. However, in the salt-sensitive wheat genotypes viz., Ibaa99 and Rabia, the reduced content of the chlorophyll was 23.6 and 25.5 SPAD respectively.

Table 2. Effect of salinity levels on the chlorophyll content (SPAD) of the five bread wheat genotypes.

Genotypes	2 ds/m	8 ds/m	16 ds/m	Means
Dijla	34.2	37.5	38.9	36.87
2H	30.8	37.4	39.5	35.90
3H	33.5	38.9	42.1	38.17
Ibaa99	33.7	29.3	23.6	28.87
Rabia	34.1	31.3	25.5	30.30
Means	33.26	34.88	3392	
$LSD_{0.05}$ Genotypes: 0.47, Salinity: 0.37, Genotypes × Salinity Interaction: 0.86				

Salinity effects on protein (%)

The results showed that overall; a decreased level of protein percentage was recorded in all the studied wheat genotypes (Table 3). However, the maximum protein percentage was detected under high salinity conditions (16 ds/m) especially in the two wheat genotypes i.e., Ibaa99 and Rabia which gave the values of 3.3% and 3.8%, respectively.

Salinity effects on K^+/Na^+ ratio

The results indicated that there were significant differences among the studied wheat genotypes for K^+/Na^+ ratio under various salinity levels (Table 4). The five genotypes showed variation and gave the highest rates at the control treatment and then gradually decreased with two salt concentrations (8 and 16 ds m^{-1}), the wheat genotypes 2H and Dijla recorded with values of 6.3 and 5.9, respectively at the high salt concentration (16 ds m^{-1}) compared with the control treatment (12.6 and 13.2), respectively. However, the two wheat genotypes Ibaa99 and Rabia provided extremely lower values at the same treatment which amounted to 0.8 and 0.6, compared to the control treatment (9.5 and 10.8).

Salinity effects on carbohydrates content (mg g^{-1})

Analysis of the data showed that there was a significant effect of salinity on carbohydrate content in the leaves of three wheat genotypes (Table 5). By increasing salinity levels from 2 to 16 dc/m, the accumulation of carbohydrate content was evident in leaves of the tolerant genotypes (2H, 3H, and Dijla) with values of 600.14, 590.60, and 560.80 mg g^{-1}, respectively. However, the carbohydrate

content was significantly decreased in the two genotypes (Ibaa99 and Rabia) by treating with a high level of salt which gave values of 163.97 and 191.8 mg g^{-1}, respectively.

Salinity effects on proline acid (µg g^{-1})

Proline accumulation is a usual response to salt stress, with increasing levels of NaCl applied from 2 to 16 ds/m, the proline content was also enhanced in the five genotypes (Table 6). Proline accumulation in leaves of wheat plants significantly increased in all the genotypes compared to the control treatments, when plants were exposed to high salinity levels (16 ds m^{-1}), and the proline acid content was significant in the tolerant wheat genotypes Dijla, 3H, and 2H with values of 24.30, 23.14, and 21.87 µg g^{-1} respectively. However, the control treatment for the two genotypes Ibaa99 and Rabia, provided the lowest values of proline acid content (4.96 and 4.56 µg g^{-1}), respectively.

Table 3. Effect of saline levels on the protein (%) of the five bread wheat genotypes.

Genotypes	2 ds m^{-1}	8 ds m^{-1}	16 ds m^{-1}	Means
Dijla	14.7	12.8	8.5	12.00
2H	13.6	11.6	5.7	10.30
3H	12.7	12.6	7.4	10.90
Ibaa99	12.9	10.0	3.3	8.73
Rabia	11.5	9.3	3.8	8.20
Means	13.08	11.26	5.74	
$LSD_{0.05}$ Genotypes: 0.12, Salinity: 0.25, Genotypes × Salinity Interaction: 0.46				

Table 4. Effect of salinity levels on the K^+/Na^+ Ratio of the five bread wheat genotypes.

Genotypes	2 ds m^{-1}	8 ds m^{-1}	16 ds m^{-1}	Means
Dijla	13.2	8.1	5.9	13.2
2H	12.6	8.7	6.3	12.6
3H	11.3	7.4	5.4	11.3
Ibaa99	9.5	3.2	0.8	9.5
Rabia	10.8	3.4	0.6	10.8
Means	11.48	6.16	3.8	
$LSD_{0.05}$ Genotypes: 0.06, Salinity: 0.11, Genotypes × Salinity Interaction: 0.17				

Table 5. Effect of salinity concentrations on the carbohydrates content (mg g^{-1}) of the five bread wheat genotypes.

Genotypes	2 ds m^{-1}	8 ds m^{-1}	16 ds m^{-1}	Means
Dijla	400.20	500.50	560.80	487.17
2H	538.12	570.70	600.14	569.65
3H	450.15	566.20	590.60	535.65
Ibaa99	370.14	269.20	163.90	267.75
Rabia	328.14	216.54	191.8	245.49
Means	417.35	424.628	421.448	
$LSD_{0.05}$ Genotypes: 17.78, Salinity: 23.27, Genotypes × Salinity Interaction: 38.18				

Table 6. Effect of salinity concentrations on the proline acid content (µg g^{-1}) of the five bread wheat genotypes.

Genotypes	2 ds m^{-1}	8 ds m^{-1}	16 ds m^{-1}	Means
Dijla	12.68	15.59	24.30	17.52
2H	11.87	13.99	21.87	15.91
3H	12.01	14.76	23.14	16.64
Ibaa99	4.96	10.34	14.89	10.06
Rabia	4.56	9.99	15.28	9.94
Means	9.22	12.93	19.90	
$LSD_{0.05}$ Genotypes: 1.28, Salinity: 1.44, Genotypes × Salinity Interaction: 1.95				

Gene expression estimation

Real Time-PCR technology was used to study the expression of the TaOPR1 gene in wheat cultivars under saline stress conditions. Both TaOPR1 and B-actin genes were amplified and the melting point was studied, and determine the value of the CT threshold cycle for them. The amplification results showed that the reaction product of the reference gene B-actin and the identification of CT values ranged between 25.8 to 12.3 in all studied models (until 15.9) while the CT values recorded for the TaOPR1 gene ranged between 31.25 to 18.5 (Table 7, Figure 1). Depending on the interaction efficiency and CT values, the value of gene expression for studied wheat models was calculated. Results further indicated the identification of the TaOPR1 gene in all studied wheat cultivars, however, the magnitude of gene expression was recorded with significant differences among these cultivars under saline treatments and control. Wheat cultivars 2H and Dijla were recorded with the highest values for expression of the TaOPR1 gene at the salinity level of 16 dS/m, with values of 6.49 and 4.0, respectively. However, that gene expression did not differ significantly (which reached 6.20) from the cultivar 3H at the same high salt concentration (16 dS/m) which amounted to 3.768, compared with the control treatments for all studied cultivars recorded with the same value (1). The two wheat cultivars Ibaa99 and Rabia gave the low and same value (1.3) for gene expression at the high salt concentration.

Table 7. Effect of salinity concentrations on the expression of TaOPR1 gene of the five bread wheat genotypes.

Genotypes	TaOPR1 and *B-actin* genes					
	EC (ds/m)	Ct of target	Ct of reference	ΔCt	ΔΔCt	Fold
Dijla	2	28.40	23.50	4.90	0.00	1.00
	8	24.52	20.72	3.80	-1.40	2.66
	16	19.04	15.90	3.10	-2.00	4.00
2H	2	25.73	21.98	3.75	0.00	1.00
	8	22.87	20.07	2.80	-1.20	2.30
	16	18.50	16.25	2.20	-2.70	6.50
3H	2	28.45	20.65	7.80	0.00	1.00
	8	25.39	18.50	6.89	-1.21	2.33
	16	18.50	12.30	6.20	-1.90	3.77
Ibaa99	2	31.25	24.10	7.15	0.00	1.00
	8	29.75	23.00	6.75	-0.40	1.30
	16	29.70	23.00	6.70	-0.45	1.30
Rabia	2	30.10	25.80	4.30	0.00	1.00
	8	29.60	25.40	4.20	-0.10	1.07
	16	29.00	25.10	3.90	-0.40	1.30

$LSD_{0.05}$ Genotypes: 0.08, Salinity: 0.06, Genotypes × Salinity Interaction: 0.14

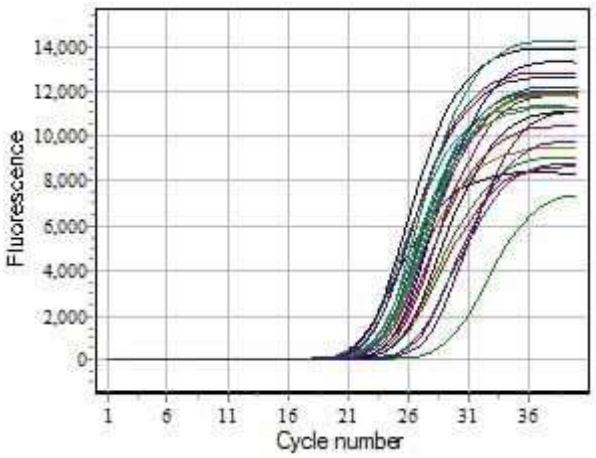

Figure 1. Real time PCR product of TaOPR1 gene and standard gene B-actin in five wheat cultivars.

DISCUSSION

With increased salinity levels (from 2 to 16 ds/m), the chlorophyll and protein content also enhanced significantly in the tolerant wheat genotypes i.e., Dijla, 2H, and 3H, while the reduced rate of the said two components was recorded in the control cultivars Ibaa99 and Rabia. These present results were having a great analogy with the findings of Al-Burki *et al*. (2019) who studied molecular performance assessment of bread wheat (*Triticum aestivum* L.) cultivars under several types of irrigation water. Rady *et al*. (2019) findings also indicated that the reduction in the chlorophyll content and protein percentage in the wheat leaves under salt stress conditions can be attributed to a decrease in photosynthesis. Salinity effect and reduces the chlorophyll content in the salt-sensitive genotypes while increasing the same in salt tolerant genotypes. Salinity harms the chlorophyll content in many crops by imposing adverse effects on chlorophyll synthesis and accelerating its decomposition, thus reducing the photosynthetic capacity (Arunyanark *et al*., 2008). The ability to maintain chlorophyll content under salinity stress is also a property of salt resistance in wheat (Cuin *et al*., 2010).

The five wheat genotypes showed significant variation in the K^+/Na^+ ratio and the highest ratio was recorded in the control treatment and then gradually decreased with two salt concentrations (8 and 16 ds m-1), and the genotypes Ibaa99 and Rabia showed extremely lower values at the same treatment. This decrease in the K^+/Na^+ ratio was due to the high concentration of sodium ions in plant tissues at high levels of salinity and its competition with potassium ions. These results were consistent with the past findings which indicated that K^+ deficiency becomes severe under salt stress, resulting in poor growth (Shabala and Pottosin, 2014)**.**

With increasing salinity levels from 2 to 16 dc/m, carbohydrate accumulation was evident in leaves of the tolerant genotypes (2H, 3H, and Rabia). In agreement with the current study results, several researchers have indicated that the tolerant wheat genotype plants accumulated higher carbohydrate content than the sensitive genotypes under salt stress conditions (Shaddad *et al*., 2013; Rady *et al*., 2019), and under salt stress, young leaves accumulated carbohydrates and dehydrin proteins to reduce sodium toxicity (Xiao *et al*., 2020). The accumulation of compatible solutes such as carbohydrates to provide protection against osmotic stress, to stabilize enzymes and desiccant membranes is one of the ways plants have evolved to deal with salinity stress implying that carbohydrate metabolism is important for salt stress tolerance (Tanji, 1990). Also, the proline accumulation significantly increased in the salt-tolerant wheat genotypes (Dijla, 3H, and 2H) leaves compared to the salt-sensitive cultivars (control treatments) after exposing the plants to high salinity level (16 ds m-1). These results got support from the studies of Rady *et al*. (2019) who found that proline content increased more under 12 ds/m NaCl than those under control, and also proline attenuated the inhibitory effects of NaCl to varying degrees, ensuring the significant improvement in biomass yield. Accumulation of proline under salt stress regulates the osmotic balance between cytosol and vacuoles (Ibrahimova *et al*., 2021), and its high content in plant tissues is an indicator of salt-induced damage (Hossain *et al*., 2019). Maurizio *et al*. (2019) reported that increasing the amount of proline in the presence of salt was related to its osmotic and antioxidant properties under stress conditions.

Concerning the gene expression, which also showed significant differences among the wheat genotypes under saline and non-saline stress conditions. Wheat cultivars 2H and Dijla were recorded with the highest values of expression of the TaOPR1 gene, while the two other cultivars viz., Ibaa99 and Rabia showed lower values for gene expression at the high salt concentration. In wheat genotypes, the over-expression of the TaOPR1 gene improves salt tolerance by regulating the ABA and ROS signaling pathways (Dong *et al*., 2013), and with that, the mRNA levels of the TaOPR1 gene were also enhanced significantly under saline stress conditions (Ejaz *et al*., 2020).

CONCLUSIONS

Wheat genotypes viz., Dijla, 2H, and 3H outperformed the other genotypes and showed the highest chlorophyll content, protein (%), carbohydrates content, and proline acid under saline stress conditions, except the ratio of K+ / Na+. In addition, the expression values of the TaOPR1 gene were the highest in the above salt tolerant genotypes compared to salt-sensitive genotypes. Therefore, the salt-tolerant wheat genotypes Dijla, 2H, and 3H were found to be a good source for future wheat breeding programs and can also be grown with salinity conditions.

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