Molecular and physiological insights of salt tolerance in hulled barley (*Hordeum vulgare* L. var. nudum)

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Background
The nutritional value of hulled barley makes it a promising resource for creating new healthy foods globally. However, improving the salt tolerance of certain barley cultivars remains a challenge, despite their inherent salt tolerance.
Objective
This study aims to investigate the physiological and molecular mechanisms associated with salt tolerance in barley, focusing on the expression of genes involved in regulating cellular ion homeostasis, detoxification, and water transport.
Materials and methods
Three barley cultivars were subjected to different levels of NaCl concentrations. Data on several growth parameters and gene expression were measured and recorded.
Results and conclusion
Increasing salinity affected shoot and root length, fresh and dry weight, depending on genotype. Giza-130 showed higher dry weight, followed by Giza-135, while Giza-136 showed the lowest value. Giza-130 exhibits the ability to regulate intracellular ion concentration through a higher expression level of the NHX1-gene, demonstrating its ability to effectively absorb water under salinity stress, due to its high expression level of the *hvpip* –aquaporin gene and effectively remove reactive oxygen species and reduces oxidative stress through the accumulation of higher concentrations of catalase, ascorbate peroxidase, glutathione S-transferase, and superoxide dismutase. In contrast, Giza-136 showed down-regulated gene expression and higher sensitivity to salt stress. Giza-130 was salt tolerant, followed by Giza-135 while Giza-136 was very sensitive. The genotype-specific regulation of gene expression not only highlights the important role of these genes in protecting plants against salt-induced oxidative stress but also improves our understanding of the salt stress tolerance of barley and plays an important role in improving salt tolerance in other crops.

Keywords:
barley, enzymes, expression, gene, oxidative, salt

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Introduction

Barley is a widely cultivated cereal crop in temperate regions and is the fourth most produced crop in the world after wheat, rice, and maize [1]. It accounts for ~8% of the world's agricultural land. Hulled barley (*Hordeum vulgare* L. var. nudum) is a variety of barley that is cultivated in East Asia, mainly on the Qinghai-Tibet Plateau at an average altitude of 4,500 m [2]. Despite its potential, barley is not widely used for human consumption and is usually grown for cultural or regional reasons where wheat is less profitable [3]. However, it is now gaining importance as a health food in Europe, North America, and other nontraditional barley-growing regions [4]. Barley grains are rich in minerals, protein, and lysine, and have high β-glucan content, which helps inhibit cholesterol synthesis [5]. Hulled barley also has advantages for the food industry as it eliminates the need for processing steps. Therefore, hulled barley is considered a valuable resource for breeding new healthy foods around the world [6]. Salt stress, a significant abiotic stress, poses a widespread challenge to plant growth and productivity. This is a result of salt accumulation in the soil, leading to reduced water and nutrient availability for plants [7,8]. Drought, salinity, heat, floods, air pollution, and climate change are some of the main causes of reduced crop growth and productivity [9]. Recent studies indicate that soil salinity is expected to increase further due to the effects of climate change [10]. Therefore, improving crop productivity through the development of salt-tolerant crop varieties becomes a strategy to mitigate

salt stress. Salt stress causes an increase in the production of reactive oxygen species (ROS), which can damage cellular components [11,12].

Plants have evolved various adaptive mechanisms to cope with salt stress, including changes in gene expression. Previous research has identified several genes involved in plant responses to salt stress, including those involved in ROS scavenging, ion transport, and osmotic regulation [13]. Understanding the regulation and role of these genes in salt stress tolerance can provide insights into developing salt-tolerant crops and improving agricultural productivity in saline environments [14]. Many studies have focused on identifying and characterizing genes involved in plant responses to salt stress, which play important roles in maintaining cellular ion homeostasis, detoxifying reactive oxygen species, and regulating carbon metabolism under salt stress conditions, such as hvpip [15], nhx1 [16–18], GSTTPS, sod, apx [19,20].

This study aimed to investigate the impact of salt stress on plant biomass production in three cultivars of hulled barley and to explore the molecular mechanisms that underlie barly's response to stress. Specifically, we examined the expression of hvpip-aquaporin, nhx1 and genes encoding antioxidant enzymes (cat, apx, sod and gst) under salt stress conditions. A comprehensive understanding is necessary for devising plans to enhance plant salt tolerance and improve food security in areas with high salt concentrations.

Materials and methods

Plant materials and saline treatments

In this experiment, three cultivars of Egyptian hulled barley were grown (Giza-130, Giza-135, and Giza-136). Cultivation was carried out in 1 L plastic pots filled with a soil mixture consisting of equal parts of sandy soil and peat moss (ratio 1 : 1 v/v). To ensure optimal growth conditions, seedlings were irrigated with 400 ml of one-tenth concentration MS solution [21] every day and placed in a greenhouse with controlled environmental conditions. To ensure adequate soil moisture, soil water tension was maintained below 60 kPa throughout the experiment. After 30 days of growth, plants were exposed to salt stress. This was achieved by gradually introducing NaCl (sodium chloride) into the nutrient solution at different concentrations: .0 mM (control), 50, 100, and 200 mM for 15 days. Throughout the experiment, the temperature in the greenhouse was kept constant at 35°C, and the photo-synthetically active radiation (PAR) was set at 2743 $\mu mol\, m^{-2}\, s^{-1}$. To ensure statistical validity and reliable results, each NaCl treatment and control group without NaCl treatment were repeated five times.

Measurement of growth

To evaluate the effects of salt stress on plant growth, shoot, and root length as well as plant fresh weight of salt treated and control plants were measured. Then, the sample was dried in a forced drying oven (Heraeus-0871, USA) at 80°C for three days. After the drying process, the dry weight of the sample is determined.

RNA extraction and real-time-PCR (qPCR) analysis

RNA extraction and quantitative polymerase chain reaction (qPCR) analysis were accomplished through the following methods. Frozen leaf tissue weighing 100 mg was used for total RNA isolation. The RNeasy Plant Mini kit (Qiagen, Germany, CAT NO.74903 and 74904) was used according to the manufacturer's instructions. Total RNA was then reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA, Cat NO. K1621). The expression level of messenger RNA (mRNA) related to salt tolerance in barley shoot tissues was measured using real-time PCR (qPCR). Maxima SYBR Green/ROX qPCR Master Mix kit (Thermo Scientific, USA, Cat NO. K0221) was used for this purpose. Primer nucleotides were designed using Primer 3 software (Table 1) and the Actin gene was used as a housekeeping gene. The PCR reaction included an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, extension at 72°C for 60 s, and a final extension at 72°C for 4 min. qPCR reactions were performed in triplicate for all genes and a Stratagene MX3000P qPCR machine was used.

Data analysis

Data analysis involved measuring the comparative Ct value (ΔΔCt), obtained by subtracting the ΔCt value of the calibrator from the ΔCt value of the treated samples. Additionally, relative expression fold changes were calculated using the $2^{-\Delta\Delta CT}$ formula of Livak and Schmittgen [22]. To determine the significance (P value < 0.05) between mean genotype differences, an independent, unpaired Student's t test was applied.

Results impact of salt stress on plant growth the effects of salt stress on shoot and root length, plant fresh and dry weight were investigated in three barley cultivars, namely: Giza-130, Giza-135, and Giza-136 under salt

Table 1 Real-time polymerase chain reaction primers used

Gene name	Sequences	
	Forward (5→3')	sReverse (5→3')
Actin	AGACCTTCAACACCCCTGCTATGT	CCAATCCAGACACTGTACTTCCTT
NHX1	TACGGTTTTCTGCCTCTGTCACA	ACAA CATCTGGTCATACTGCCG
HvPIP2:1	GCTAGCTTAGCAATGGCCAAGGAC	GTCGGACTGGTGCTTGTACC
Gst	AGCATCTCGTCAGAAACCCGT	TCCTTCAGGTTGCCCTCTCTT
Sod	CCGAAGATGAAATCCGCCAT	CGGCCAATGATTGAATGTGG
Cat	CGACGACAAGATGCTGCAGT	TGGTTGTTCTTGAAGCCGC
Apx	CGGAGCTTTTGAGTGGTGACA	CCGCAGCATATTTCTCCACAA

stress conditions. Shoot length is an important indicator of the plant's ability to cope with salt stress. In the case of Giza-135 and Giza-136, the leaves turned yellow and eventually died 2 weeks after exposure to salt stress, unlike the control plants which remained green (Fig. 1). The results showed that shoot length decreased significantly in all three cultivars when increasing NaCl concentration. Giza-130 is more tolerant to salinity stress while Giza-136 exhibited the highest sensitivity to salt stress, with the greatest reduction in shoot length at the highest NaCl concentration of 200 mM (Table 2 and Fig. 1). Salt stress also has a significant impact on plant fresh weight. The results showed that plant fresh weight decreased significantly as NaCl concentration increased for the three cultivars. The Giza-136 showed the highest sensitivity to salt stress, with the greatest reduction in fresh weight at the highest NaCl concentration of 200 mM. Similarly, salt stress affected plant dry weight in all cultivars, with variations depending on genetic background (Table 2). The cultivar Giza-130 showed a less pronounced decrease in dry weight, followed by Giza-135, while the cultivar Giza-136 showed the most significant decrease in dry weight under salt stress conditions.

Figure 1

The effect of salinity stress on plant growth of three hulled barley cultivars. A: Giza-130, B: Giza-135 and C: Giza-136.

Expression of genes encoding antioxidant enzymes (SOD, GST, CAT and APX)

The changes in *sod* gene expression under salt stress in hulled barley cultivars was examined Fig. 2a. At 50 mM NaCl, Giza-135 accumulated higher levels of *sod* transcripts compared with other cultivars. As NaCl concentration increased up to 100 mM, expression levels increased, with Giza-130 showing a 9-fold increase compared with a 7-fold increase in Giza-135. However, in Giza-136 it only reached a 2-fold increase at the same concentration. At 200 mM NaCl, *sod* expression levels were significantly decreased in all cultivars, with a significant decrease observed in Giza-136.

The expression of the *gst* gene was affected by salt stress, and an increase in expression was observed when the NaCl concentration increased to 100 mM. Giza-130 showed a 10-fold increase in expression compared with 3-fold increase in Giza-135, while Giza-136 down-regulated *gst* expression at the same concentration. At 200 mM NaCl, Giza-130 maintained a higher expression level compared with Giza-135, and Giza-136 showed a negative expression level under salt stress (Fig. 2b).

In this study, *cat* gene expression was investigated in different cultivars, and the results indicated that Giza-130 exhibited significantly higher levels of expression at

Table 2 The effect of salt stress on biomass production in three barley cultivars

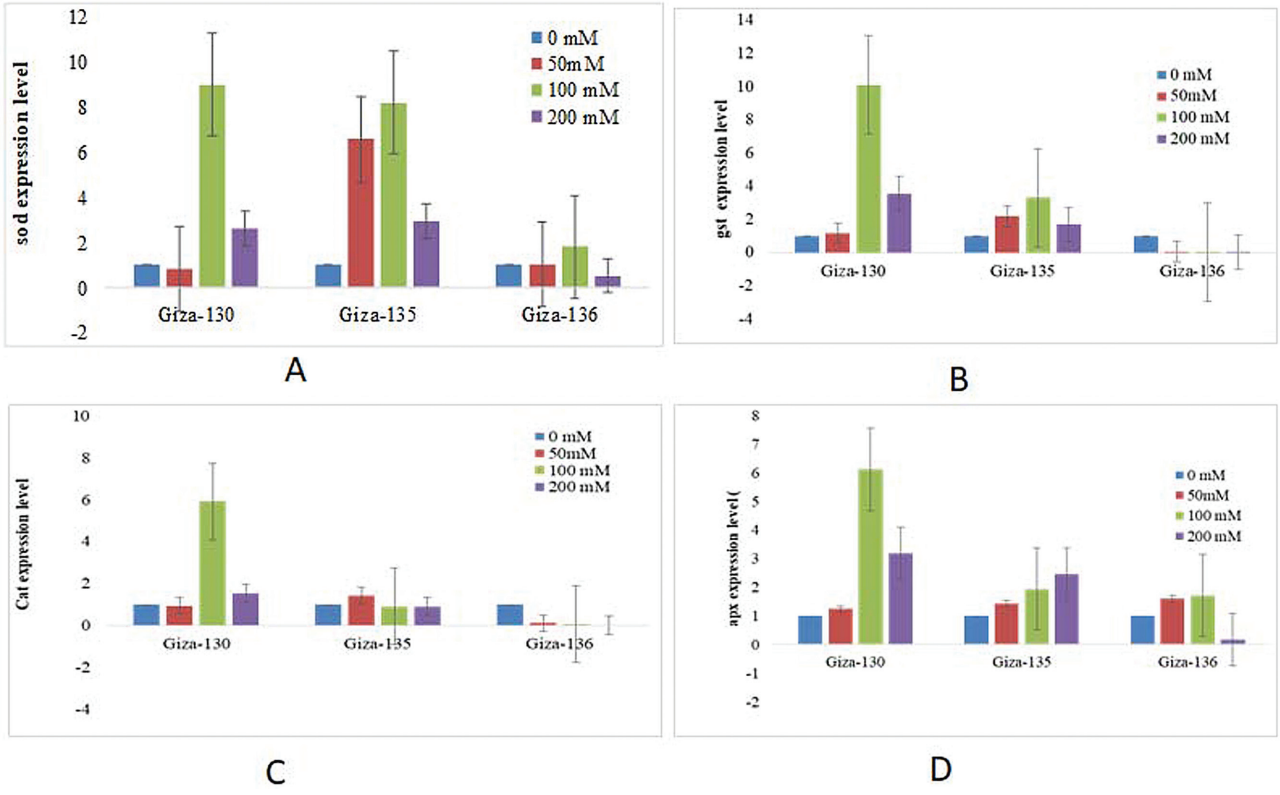
Cultivars	NaCl Treatment (mM)	Shoot length (cm)	Root length (cm)	Whole plant (cm)	FW (g)	DW (g)
Giza-130						
	0	25±1.1	14±1.2	39	5.72±0.2	5.12 (100%)
	50	26±2.0	18±1.5	44	6.21±0.1	5.49 (107%)
	100	27±1.4	17±1.3	44	5.46±0.2	4.80 (93%)
	200	25±1.2	17±1.4	42	4.41±0.3	3.81 (74%)
Giza-135						
	0	28±1.4	14±1.5	42	3.79±0.1	3.37 (100%)
	50	27±1.4	17±1.7	44	4.58±0.2	4.07 (120%)
	100	21±1.8	15±1.3	36	3.22±0.2	2.87 (85%)
	200	15±3.0	16±1.3	31	2.87±0.2	2.73 (69%)
Giza-136						
	0	25±2.1	15±1.1	40	5.78±0.3	4.97 (100%)
	50	27±2.0	17±1.2	44	5.42± 0.1	4.72 (94%)
	100	22±1.1	15±1.4	37	4.60±0.1	4.04 (81%)
	200	14±1.1	14±1.3	28	2.60±0.1	2.13 (42%)

Data are mean of five replicates±SE.

100 mM NaCl compared with other cultivars tested at the same salt concentration. However, expression levels decreased significantly with an increase in NaCl concentration in all cultivars, as shown in Fig. 2c.

The effect of salt stress on *apx* gene expression in different hulled barley cultivars was investigated, and the results presented in Fig. 2d. Increasing the salt concentration from 50 to 100 mM increased *apx* gene expression. It was observed that, Giza-130 showed the highest expression level (6-fold increase) compared with other cultivars at the same salt concentration. However, increasing the salt concentration to 200 mM led to a significant decrease in *apx* gene expression. Giza-130 showed higher expression levels (3-fold increase), followed by Giza-135 (2-fold increase), while the expression was completely inhibited in Giza-136 at 200 mM NaCl.

Figure 2



The expression of genes encoding antioxidant enzymes in hulled barley under salinity stress conditions: A-*sod*, B-*gst*, C-*cat* and D-*apx*.

Expression of genes associated with water transport and ion homeostasis in barley under salt stress

In this study, the impact of salt stress on the *HvPIP* gene, which plays a crucial role in water transport activity and regulation of barley PIP aquaporins, was investigated in three different hulled barley cultivars. The results represented in Fig. 3 reveal that the folding of the *HvPIP* transcript is enhanced under salt stress conditions. At 50 mM NaCl, Giza-130 exhibited a 5-fold increase in expression, while Giza-135 and Giza-136 showed 4 and ~3-fold increases, respectively. Furthermore, increasing NaCl concentration from 50 mM to 100 mM resulted in increased expression of *HvPIP* gene, with Giza-130 exhibiting the highest increase, followed by Giza-135, and Giza-136 showing the lowest expression. However, at high salinity levels (200 mM), *HvPIP* expression levels decreased in all cultivars, with Giza-135 exhibiting the greatest decrease and Giza-130 maintaining relatively higher expression levels.

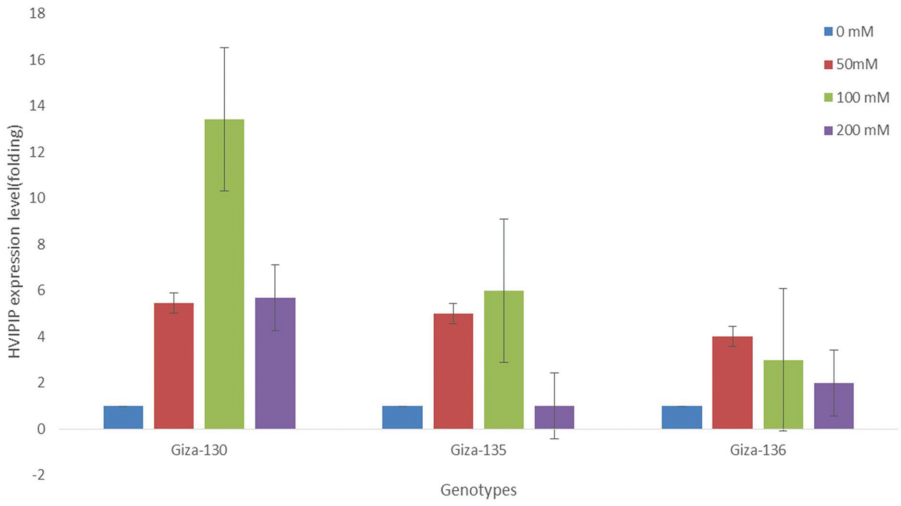
In this study, we examined *Nhx1* gene expression levels in three different cultivars of hulled barley under salt stress. According to the data shown in Fig. 4, *Nhx1* expression levels increased with increasing salt concentration. For example, increasing NaCl concentration from 50 mM to 100 mM increased the expression levels from 1.5-fold to 6-fold in Giza-130 and from 2-fold to 7-fold in Giza-135. However, in Giza-136, the expression was down-regulated even at all salt concentration. A higher salt concentration of 200 mM decreased expression levels in all cultivars. Giza-136 showed a significant decrease compared with Giza-130, which maintained higher expression levels, followed by Giza-135 at the same salt concentration.

Discussion

Salt stress is a well-known abiotic as a major abiotic stress factor that negatively affects the growth, development, and yield of crops through ionic and osmotic stress [7,8,23]. In this study, seedlings of three hulls barley cultivars were exposed to saline stress for 15 days. Shoot length is an important indicator of a plant's ability to withstand salt stress. Increased NaCl concentrations significantly decreased shoot and root length in all three cultivars. Salinity stress also led to a reduction in plant fresh and dry weights, but the extent of the reduction varied among cultivars. Dry weight loss was minimal in Giza-130, followed by Giza-135, and Giza-136 showed the most significant reduction. These results show that Giza-130 has better salt tolerance than other cultivars, while Giza-136 is more sensitive.

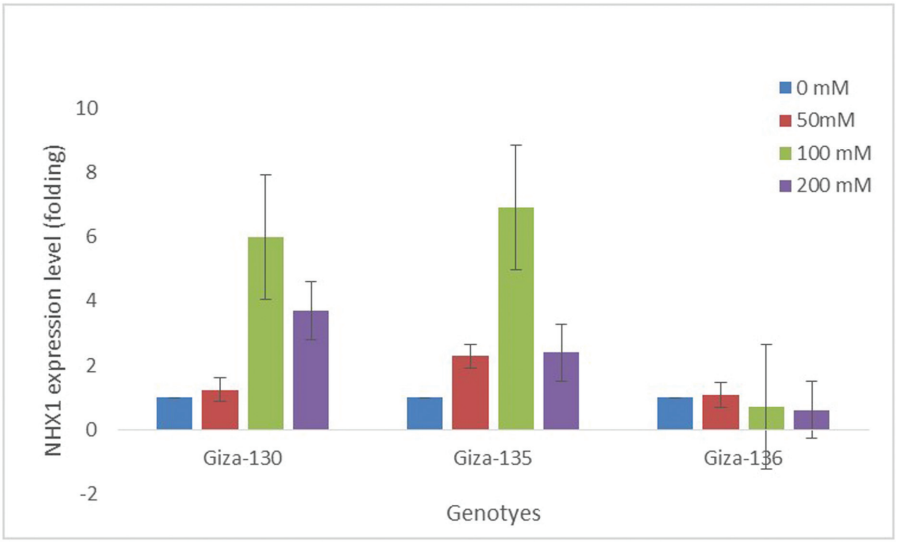
Plants have developed various mechanisms, including regulation of gene expression, to cope with salt stress conditions. Several genes such as *hvpip, nhx1, cat, gst, sod,* and *apx* have been identified to play a role in plant response to salt stress [7,9]. *gst* is involved in detoxifying ROS in plants under stress conditions, including salt stress [24,25]. This study found that *gst* gene expression was upregulated under salt stress and Giza-130 showed the highest increase in expression levels compared with Giza-135 and Giza-136. These findings suggest that the salt tolerance observed in Giza-130 is due to its ability to accumulate higher levels of *gst* expression in response to salt stress. The results also showed that *sod* gene expression was upregulated under salt stress, Giza-130 showed a higher increase in expression levels

Figure 3



The effect of salinity stress on *hvpip* gene expression of hulled barley cultivars.

Figure 4



The effect of salinity stress on *nhx1* gene expression of hulled barley cultivars.

compared with Giza-135, in contrast, the expression in Giza-136 significantly decreased at 100 mM NaCl. These findings suggest that the response of barley cultivars to salt stress varies depending on their *sod* gene expression levels, which play a crucial role in protecting plants from oxidative stress caused by high salt concentrations, which in agreement with previous studies [26].

Cat and *apx* gene products are involved in detoxifying ROS in plant cells under salt stress conditions [19]. The results showed that *cat* gene expression was significantly higher in Giza-130 compared with other cultivars at 100 mM NaCl, but decreased significantly with an increase in salt concentration in all cultivars. *apx* gene expression was also upregulated at 100 mM, with Giza-130 showed the highest expression level (6-fold increase) compared with other cultivars at the same salt concentration. However, increasing the salt concentration to 200 mM led to a significant decrease in *apx* gene expression, with Giza-130 showing higher expression levels (3-fold increase), followed by Giza-135 (2-fold increase), while it was completely inhibited in Giza-136. These findings suggest that *cat* and *apx* play crucial roles in protecting plants from oxidative stress caused by high salt concentrations and that the response of Hulled barley cultivars to salt stress varies depending on their *cat* and *apx* gene expression levels. These results agreed with previous studies [27].

The effect of salt stress on *hvpip* agene involved in water transport activity was investigated, in three Hulled barley cultivars. Under salt stress conditions, *hvpip* transcriptional folding was enhanced, Giza-130 showing the greatest increase in expression at 50 mM NaCl. Increasing NaCl concentration from 50 mM to 100 mM increased *hvpip* gene expression, with Giza-130 showing the greatest increase, consistent with previous studies [28,29].

Nhx1 gene is involved in the compartmentalization of sodium ions within plant cells, essential for maintaining cellular ion homeostasis under salt stress conditions. *nhx1* is upregulated in plants subjected to salt stress [30] and participate in K+ homeostasis, endosomal pH regulation, and salt tolerance in Arabidopsis [31]. The results showed that *nhx1* expression levels increased with increasing salt concentration in Giza-130 and Giza-135, but were down-regulated in Giza-136 at the same salt concentration. These findings suggest that the *nhx1* gene plays a role in sequestering Na+ in roots via vascular Na+/H+ antiporters, limiting the transport of toxic Na+ to shoots. Giza-130 maintained higher expression levels and showed better growth under high salinity conditions compared with Giza-136, which showed impaired *nhx1* gene expression at high salt concentration.

Conclusion

Genes involved in the regulation of cellular ion homeostasis, ROS detoxification, and water transport in salt-stressed barley plants were investigated. The results of this study show that the expression levels of these genes vary between cultivars, with Giza-130 generally exhibiting higher expression levels and growing better under high salinity conditions than Giza-136. The results show that, these genes play an important role in protecting barley plants against oxidative stress caused by high salt concentrations and that different hulled barley cultivars have different

abilities to cope with salt stress. These results could be useful for breeding programs aimed at developing salt-tolerant cultivars and improving crop productivity in saline environments.

Authors' contributions

MHS and AHF planned the experiments. NSS carried out field and laboratory sample preparations and experiments. All authors contributed to the interpretation of the results. NSS took the lead in performing the research, analyzing data, and writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Nil.

Conflicts of interest

The authors declare that they have no conflict of interest.

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