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ORIGINAL PAPER

# Differential Gene Expression Responses to Salt and Drought Stress in Tall Fescue (*Festuca arundinacea* Schreb.)

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## Abstract

Understanding gene expression kinetics and the underlying physiological mechanisms in stress combinations is a challenge for the purpose of stress resistance breeding. The novelty of this study is correlating the physiological mechanisms with the expression of key target genes in tall fescue under a combination of various salinity and osmotic stress treatments. Four drought- and salt-responsive genes belonging to different crucial pathways evaluated included one transcription factor *FabZIP69*, one for the cytosolic polyamine synthetase *FaADC1*, one for ABA signaling *FaCYP707A1,* and another one for the specific $Na^+/H^+$ plasma membrane antiporter *FaSOS1* involve in osmotic homeostasis. *FaSOS1, FaCYP707A1,* and *FabZIP69* were induced early at 6 h after NaCl treatment, while *FaSOS1 and FaCYP707A1* were transcribed gradually after exposure to PEG. However, stress interactions showed a significantly increased expression in all genes. Expression of these genes was positively correlated to Pro, SSs, IL, DPPH, and antioxidant enzyme activity and negatively correlated with RWC, total Chl, and MSI. Chemical analyses showed that tall fescue plants exposed to the combination of stresses exhibited increased quantity of reactive oxygen species ($H_2O_2$), EL and DPPH, and higher levels of antioxidant enzyme activities (CAT, and SOD), Pro, and SSs content, compared with control seedlings. Under dual-stress conditions, the expression of *FabZIP69* was effective in controlling the expression of *FaSOS1* and *FaADC1* genes differently.

**Keywords** Abiotic stress · Chlorophyll · DAB staining · Gene expression · Proline

## Abbreviations

| | |
|---|---|
| ROS | Reactive oxygen species |
| ABA | Abscisic acid |
| CAT | Catalase |
| DMSO | Dimethyl sulfoxide |
| Car | Carotenoids |
| Chl | Chlorophyll |
| C | Electrical conductivity |
| IL | Ion leakage |
| MSI | Membrane stability index |
| NTC | No template controls |
| Pas | Polyamines |
| PEG | Polyethylene glycol |
| Pro | Proline |
| Put | Putrescine |
| RWC | Relative water content |
| SRA | Sequence read archive |
| SOD | Superoxide dismutase |
| TF | Transcription factor |
| SSs | Total soluble sugar |
| DAB | 3,3-Diaminobenzidine |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |

## Introduction

The critical challenge for plant survival when dealing with altering environmental conditions is to endure abiotic stresses mostly in combination [1]. Plant growth and development are significantly hampered by drought and salt stress, resulting in production losses and plant death [2]. Plants have established a series of intricate cellular signaling pathways like mitogen-activated protein kinase (MAPK) cascades, reactive oxygen species (ROSs), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) to avoid damages instigated by these prevailing stresses [3]. These signaling pathways and their crosstalk activate transcription

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factors like bZIP, HSF1, SRRA/SKN7/PRRr1, PCR1, and MYB to regulate downstream corresponding genes [4] to make the necessary adjustments and generate functions to cope with the stressors. Understanding the associated core set of genes and traits to develop stress-tolerant and high yielding species is inevitable [5]. Drought is a multi-aspect stress in plants that affects various dimensions at morphological, physiological, and molecular levels [6]. Likewise other stresses, drought stress also induces the formation of ROSs and leads to the condition of oxidative stress [7]. Dehydration or osmotic stress results in a variety of biochemical and physiological shifts in plants, notably a decrease in photosynthesis and $CO_2$ fixation, a build-up of osmo-protectants, and adjustments in carbohydrate metabolism [8].

Salinity or precisely higher levels of $Na^+$ and $Cl^-$ generates ionic and osmotic stress at the same time [9]. The transduction mechanisms for ionic and osmotic stress responses are anticipated to be complex, involving a variety of signal molecules such as ABA, cyclic nucleotides, and inositol polyphosphates [8]. As a result, the specific pathway(s) by which plants adopt to salt and drought is still in doubt. At the molecular level, however all the modifications are almost certainly the result of changes in the expression of genes. Thus, identifying the key genes and understanding their regulation in response to drought and/or salt stress.

*Festuca arundinacea*, belonging to the Pooideae subfamily, share large-scale synteny or a close evolutionary relationship to perennial ryegrass, *Brachypodium*, wheat, and rice [10, 11]. *Festuca arundinacea* is an economically important cool-season turfgrass and a principal forage that is widely planted in transitional regions and frequently used as a model plant to identify molecular mechanisms of stress tolerance in perennial grasses [12]. Tall fescue is also an allohexaploid with a large genome size [13] and up to now its large nuclear genome has not been sequenced completely (Based on NCBI, EBI, ENSEMBLE, and DDBJ databases), while there are some unpublished de novo genome assembly from tall fescue. Therefore, the genome sequence of tall fescue is evolutionarily relevant in the Pooideae subfamily and could provide reference for studying the molecular aspects of tall fescue [14].

As a trace element, sodium can disrupt several physiological and biochemical processes, which can be detrimental to both single cells and the entire plant. Therefore, effective $Na^+$ extrusion out of the cells is essential for plants to survive and adapt to salt stress. SOS1, a plasma membrane $Na^+/H^+$ antiporter, regulates $Na^+$ efflux from cells [15] and SOS1 downregulation can cause an abnormal accumulation of $Na^+$ and loss of the salt tolerance characteristic [16]. Polyamines (PAs) are involved in different aspects of plant abiotic stress adaptation [17]. Studies have demonstrated that inducing polyamines may have protective benefits on cells or organisms that are being stressed by ROS [18], excessive temperature [19], pH [20], or osmotic stress [21, 22]. Genetically elevation of polyamine-synthesizing enzymes like ADC and ODC led to Putrescine (Put) accumulation and increased tolerance to stress conditions [23]. When plants are subjected to various stressors, such as dehydration, their ABA content rises [24]. Endogenous ABA content in plants is the action core responding to stress and is determined by the equilibrium of ABA synthesis and catabolism [25]. Cytochrome P450 CYP707A1 gene is involved in the ABA 8-hydroxylation pathway, which is the initiation of the main ABA catabolic pathway [24]. bZIP transcription factor (TF) family has been studied in numerous plants for instance in transgenic *Arabidopsis* and cotton plants, a bZIP TF (*GhABF2*) dramatically improved salt and drought stress tolerance and its suppression regulated the transgenic cotton to be sensitive to salt and water stress [26, 27].

The aim of this study was to evaluate the impacts of single and dual drought and salt stresses on the expression patterns of key stress-responsive genes in tall fescue in order to pave the breeding way. The novelty of the current paper is to measure molecular, biochemical, and morpho-physiological response of tall fescue under a combination of stressors. Therefore, 4 drought- and salt-responsive transcripts were selected to observe their role in water and salt stress response. Transcripts from ABA signaling pathways (*FaCYP707A1*); osmo-protectant pathways (*FaADC1*); membrane intrinsic protein (*FaSOS1*); and drought- and salt-related TF (*FabZIP69*) were selected.

## Methods

### Materials and Equipment

*Festuca arundinacea* Schreb. 'Talladega' was used as plant material. Surface-sterilized (5% v/v NaClO for 5 min) and stratified (48-h cold stratification) seeds were germinated in autoclaved soil at 24 °C. Seedlings were carefully taken out from the soil pots, rinsed with $dH_2O$ and then transferred to hydroponic culture buckets containing half-strength Hoagland solution [28]. The solutions were changed every 3 days regularly. Plants were grown in a growth chamber maintained at 24 °C/18 °C, 2000 lx light intensity 14-h/10-h photoperiod. Plants were allowed to develop 2–4 plants over 4 weeks.

### Salt and Drought Stress Treatments in Hydroponic System

After 4-weeks, tall fescue plants were subjected to the test at three levels of drought (0%, 5% and 10% (w/v) polyethylene glycol (PEG, 10,000), and three levels of salt stress (0-, 200-, and 400-mM NaCl). After imposing the stress treatments,

the second and third mature leaves were harvested at five designated time points (0, 6, 12, 24, and 48 h) to measure the mRNA level of *FaSOS1*, *FabZIP69*, *FaADC1*, and *FaCYP707A1*. Osmotic pressure of each solution was measured using an osmometer (Vogel OM 802-D) which is provided in Supplementary Table S1. NaCl and PEG amounts were half strength in Hoagland solution and seedlings grown in absolute half-strength Hoagland solution were employed as untreated control plants.

## Gene Expression

### Total RNA Isolation and cDNA Synthesis

For extracting genomic RNA, 100-mg fresh mature leaf samples were homogenized in 1-ml Trizol reagent using sterilized mortar and pestle with liquid nitrogen (Yekta Tajhiz Azma, Iran). After the homogenization process, the debris from the homogenate were eliminated by spinning in a centrifuge at a force of 12,000×*g* for 10 min at 4 °C. Then the supernatant which contains RNA were transferred to fresh Eppendorf tubes where 200 µl were added to it and the shacked vigorously following by incubation for 5 min at room temperature. Afterward the samples were centrifuged for 10 min in the same way. Then the upper aqueous phase were carefully transferred into a fresh tube where RNA was precipitated by adding 500-µl cooled isopropyl alcohol and inverting the tubes. Subsequently, after 30-min incubation at 4 ℃ and centrifugation for 10 min at the same conditions, the supernatant was discarded carefully. Then the invisible RNA pellet at the bottom of microtubes were washed with l-ml 70% ethanol and then the samples were centrifuged for 2 min in the same way. Then, the RNA pellets were air-dried partially for 10 min and dissolved in 10-µl RNase-free water. Respectively, RNA integrity (existence of 28s and 18s ribosomal RNAs) and quality were checked with 1% agarose gel and spectrophotometric analysis with a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA) (Fig. 1S). In order to eliminate the interference of genomic DNA, total RNA was treated with RNase-free DNase I (Yekta Tajhiz Azma, Iran) according to the manufacturer's instruction with modifications such as reducing the enzyme incubation phase to 15 min. Afterward, single-strand cDNAs were synthesized using the Easy™ cDNA Synthesis Kit (Pars Tous, Iran) containing Thermostable H-minus MMLV reverse transcriptase according to the manufacturer's instructions. cDNA Synthesis reaction was carried out in10 µl volume containing 3-µg DNase-treated total RNA as template, 5-µl buffer mix (Pars Tous 2X), and 1-µl enzyme mix. For cDNA synthesis, the following thermocycler protocol was used: 10 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C. Using spectrophotometry, cDNAs were quantified and diluted in double distilled water to 100 ng/l.

### Primer Design for Amplifying the Candidate Genes

RNA-seq data deposited at SRA/NCBI database from tall fescue were used to find candidate gene sequences to design primers. Therefore, RNA-seq data from two recent studies on tall fescue under salt and drought stress were consulted and were BLASTed against the sequences of candidate genes in rice (OsSOS1, OsCYP707A1, OsbZIP69, and OsADC1) and then after obtaining each of the corresponding gene contigs, four primer sets were designed based on the conserved domain frame of obtained contigs from tall fescue SRA database using Primer3 online software. Applied primer sets and corresponding annealing temperature are provided in Table S2.

### Analysis of qRT-PCR

SYBR Green master mix/No ROX (Amplicon) and the Real-Time Detection System (Bio-Rad CFX96, United States) were applied for conducting qRT-PCR analysis. qRT PCR amplification carried out in a 12-µl reaction volume containing 6 µl of the SYBR Green master mix, 1 µL of each primer (10 µM), and 70-ng template cDNA. All treatments had been tested three times biologically and two times technically. Threshold and baseline values of samples were calculated by the CFX manager software (Bio-Rad). The $2^{-\Delta\Delta Cq}$ method was used to determine the fold change of gene expression which is described in detail by Bustin and Nolan (2005) using alpha-tubulin as reference gene [29, 30]. The PCR efficiency for each gene was calculated using the slope of the amplification curve of five dilution series for each gene based on the following formula: % Efficiency = $(10^{-1/\text{slope}} - 1)100$ (Table S3). The no template controls (NTC) were tested in order to check DNA contamination and primer self-complementary formations that may cause false-positive results. Stress-induced and control treatments were considered as positive and negative control to determine the specificity of the assays. No fluorescence signal was detected in the NTC microtubes. However, in the microtubes with positive template, high fluorescence signals were detected, confirming that the assay was highly specific for the detection of candidate genes in the samples.

## Physiological and Biochemical Parameters

Physiological and biochemical parameters were measured using 4-week-old plants, 48 h after exposure to salt and drought stress. Plant shoot samples were excised and part of the samples were kept in a − 80 °C freezer and some were dried out for 48 h in a 70 °C oven for further measurements.

### RWC

For relative water content (RWC) assay, the third mature leaves were weighed (FW) and incubated in water for 12 h and then leaves turgid weight (TW) were measured as well. Next, dry weight (DW) of samples were measured after drying at 70 °C for 2 days [31] and RWC was estimated by means of the following formula, RWC (%) = [(FW − DW)/(TW − DW)] × 100%.

### Photosynthetic Pigments

Fresh shoot samples (0.1 g) were obtained from plants in test buckets and immediately were placed in dimethyl sulfoxide (DMSO) vial (8 ml) and then incubated to extract the pigment content (65°C, 24 h). Finally, the extract liquids were transferred to a new vial and the volume raised up to 10 ml with DMSO [32]. Total chlorophyll (Chl) (summation of Chla and Chlb contents) was quantified by measuring the absorbance of the extract liquid at two wavelengths of 663 nm and 645 nm via the spectrophotometer (Unico 2100/Vis/UV–Vis), using the following formula:

$$\text{Total Chlorophyll (mg/g FW)} = [20.3(A645) + 8.02(A663)]\ \text{V}/1000\text{W},$$

where $V$ is the final solution volume in ml and $W$ is tissue fresh weight in g.

### Proline

Proline (Pro) content was evaluated spectrophotometrically following the method described by Bates et al. [33], in such a way that, 0.5 g of fresh leaf samples were homogenized in aqueous sulfosalicylic acid [10 ml, 3% (v/v)] and incubated for 48 h. After filtering the extract with filter paper, ninhydrin reagent (2 ml) and acetic acid (2 ml) and were added to the supernatant (2 ml) and mixed in a test tube and then incubated in a hot water bath (100 °C, 1 h). Samples were transferred onto ice and after cooling, 4 ml of toluene was added to each reaction solution. The solutions were then shaken for 20 s. The absorbance measurements (520 nm) of the sample, toluene as control and standard concentrations of L-proline (Merck) with the spectrophotometer (Dynamica, Livingston, UK) were used to quantify the proline content [33].

### Total Soluble Sugar Content (SSs)

According to a method developed by Yoshida et al. (2018), the total sugar content of shoots was measured by adding 2 ml of 80% ethanol to 100 mg of the dried ground shoot [34]. After incubation at 4 °C overnight, the mixture was centrifuged at 3000 rpm for 10 min; and in case of encountering dark green color, repeating this extraction once more on the supernatant was needed. Then the mixture was boiled for 5 min after adding 10-ml anthrone. After cooling the mixture on ice, SSs was measured by reading the absorbance at 630 nm. Standard concentration series of dextrose (DNAbiotech) were used to quantify the optical densities.

### Ion Leakage (IL)

For ion leakage (IL) and membrane stability index (MSI) quantity, control, and treated leaves (0.1 g) were harvested, washed, and cut into small segments. After that, leaf disks were immersed in deionized water (8 ml) and incubated overnight, and the electrical conductivity (C1) was measured using a conductivity meter (Model Ohm-419). Subsequently, the samples were autoclaved for 20 min, at 120 °C. After chilling at room temperature, the electrical conductivity (C2) was recorded again. The IL was calculated using the following formula [35]: IL (%) = (C1/C2) × 100.

### Antioxidant Enzyme Activity

**Enzyme Extract Preparation** To prepare enzyme extract, fresh aerial tissue (100 mg) was weighed and homogenized in extraction buffer (1 ml) [100 mM potassium phosphate, 0.1 mM EDTA, and 1% polyvinylpyrrolidone (PVP), pH = 7.8] using a mortar and liquid nitrogen. Then, the extract was centrifuged (13,000 rpm, 4 °C, 30 min). Enzyme assays were performed using the supernatant using a spectrophotometer (Model Epoch Biotech, Germany) in three replicates [36].

**Superoxide Dismutase (SOD) Enzyme Activity** The activity of SOD enzyme was measured by evaluating its ability to prevent the photoreduction of nitroblue tetrazolium chloride (NBT) at 560 nm using the following formula [37]. 1 unit of SOD enzyme activity is considered to be the amount of enzyme that can prevent up to 50% photoreduction of NBT.

**Catalase (CAT) Enzyme Activity** To measure the CAT activity, the absorbance of 1 ml of the reaction mixture, which included potassium phosphate buffer, hydrogen peroxide (1M), and enzyme extract, was measured at 240 nm based on the amount of $H_2O_2$ decomposition at this wavelength [36].

### DPPH Radical Scavenging Assay

To assess total antioxidant activity, free radical scavenging assay with 2, 2-diphenyl-1-picrylhydrazyl (DPPH), (95%, Sigma-Aldrich) was applied. Frozen leaf samples (0.5 g) were pulverized and kept in 2-ml ethanol (70%) on shaker for 24 h. Subsequently, the mixture of filtered extract and DPPH (100 ml, 0.1 mM, methanol as the organic solution) was stored in the dark (room temperature, 30 min) and then the mixture's absorbance at 517 nm were measured via UV–Visible Spectrophotometer (Model Epoch Biotech, Germany) [35]. The potential ability of extracts to scavenge free radicals is calculated using the following formula:

$$\text{DPPH scavenging capacity}\,(\%) = \left[(\text{blank OD} - \text{sample OD})/\text{blank OD}\right] \times 100.$$

### $H_2O_2$ Content

To measure the amount of endogenous hydrogen peroxide ($H_2O_2$), 0.5 g of plant tissue was crushed, mixed with 0.1% (w/v) TCA, and centrifuged at 12,000×*g* for 15 min. The supernatant was mixed with 1-M potassium iodide solution and 0.1-mM potassium phosphate buffer (pH 7.0) and then were incubated at room temperature. Absorbance was measured at 390 nm using a UV–Vis Spectrophotometer (Thermo Electron Corporation), and $H_2O_2$ content was measured using the standard curve [35].

### Histochemistry DAB Staining

The stressed plant leaves were washed well with distilled water. Small segments of the leaves were cut and stained with 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, D8001). In such a way that leaf samples were added into 2 ml of the DAB staining solution (1 mg/ml, 10-mM $Na_2HPO_4$) and kept on shaker for 6 h to allow the DAB absorbed into the sample. Because of the light sensitivity of DAB, the sample tubes were wrapped with aluminum foil. After staining, to remove Chl and free DAB, leaves were kept dipped into the wash solution (ethanol:acetic acid:glycerol = 3:1:1) on shaker over night at room temperature. Then, the leaves were transferred to a fresh wash solution and observed under the binocular stereoscope [38].

### Statistical Analysis

The experiment was conducted based on a completely randomized design (CRD) with factorial arrangements (3 drought levels, 3 salt levels, and 3 harvesting times) and three replicates. qPCR analyses were performed through 7 runs. Negative and positive controls were included withing each run. Analysis of variance (ANOVA) was used to analyze the data, and the least significant difference (LSD) test was used to compare the means ($p \leq 0.05$). The obtained data were analyzed using the SAS software (9.4), and graphs were sketched using GraphPad Prism 9.

## Results and Discussion

### Gene Expression

A plethora of responsive genes to distinct drought and salt stress conditions have been characterized in tall fescue[39–47]; however, the simultaneously dual drought and salt stress aspects in tall fescue remains ambiguous. As seen from the experiments in this study, we wanted to evaluate the regulation kinetics of the candidate responsive genes under single and dual drought and salt stress conditions followed by evaluation-related morpho-physiological parameters. We detected expression kinetics of four genes which included one ABA-dependent catabolic enzyme, *FaCYP707A1,* and *FabZIP69* encoding for a bZIP type TF, and *FaADC1* encoding arginine decarboxylase involving in polyamine cycles and *FaSOS1* encoding $Na^+/H^+$ transmembrane antiporter protein. As plants are under salt stress, $Na^+$ initially reaches the cytoplasm of the cell, disrupting cellular molecular, biochemical, and physiological processes that limits plant development and alters ion homeostasis [48]. Under salt stress, after phosphorylation of SOS2–SOS3 complex, SOS1 or plasma membrane $Na^+/H^+$ ion exchangers become active to counteract this condition [49] and limiting $Na^+$ transport. Our results indicated that *FaSOS1* expression increased instantly after 6 h of NaCl treatments and its kinetics remains high for the next 48 h of stress; however, *FaSOS1* expression gradually after 12-h exposure to PEG started to increase. However, even in the absence of NaCl and PEG stressors, tall fescue plants contain *FaSOS1* transcripts, but NaCl and PEG treatments increase its levels. The *FaSOS1* gene was upregulated under moderate and severe salt stress according to the transcript level (Fig. 1). These findings demonstrate that the SOS pathway is functional for controlling $Na^+$ efflux in tall fescue. A quick response to NaCl treatment was the increased *FaSOS1* expression in leaves, which may have helped reduce the amount of $Na^+$ in cytoplasm and maintain ion homeostasis. The key mechanisms for salt tolerance are to extrude $Na^+$ from the cells and compartmentalization of $Na^+$ into the vacuoles of leaf cells through $Na^+/H^+$ ion transporters [50]. Expression kinetic of *FaSOS1* is somehow similar to NHX1 expression as a vacuolar $Na^+/H^+$ antiporter, which is increased gradually by increasing salt concentration, while its expression level hardly changed over time [50]. In a study on cotton treated with 400 mM NaCl for different time points, the relative expression of *GhNHX1* that

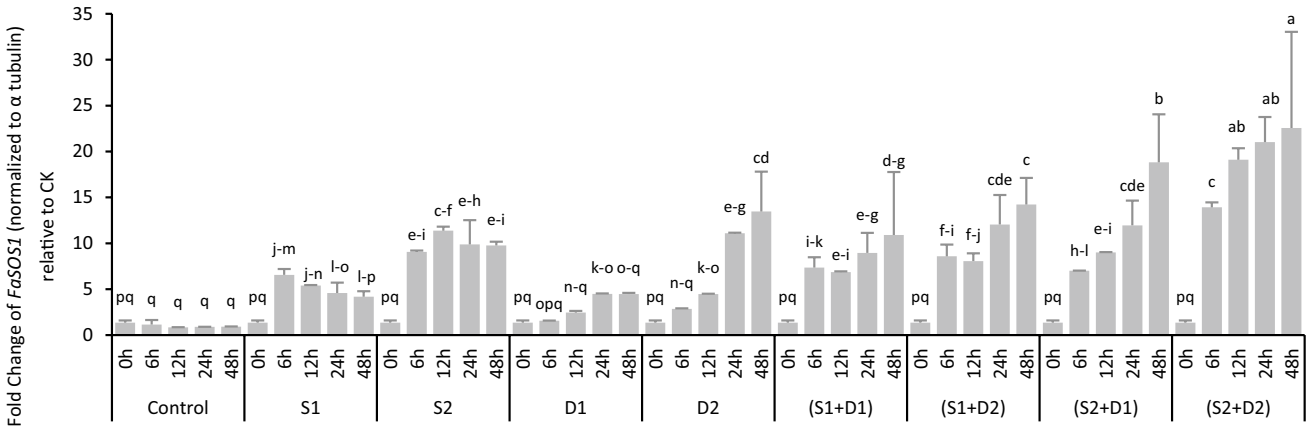


**Fig. 1** RT-PCR analysis of the expression of the *FaSOS1* gene in mature tall fescue leaves in half-strength Hoagland solution (Control), 200-mM NaCl (S1), 400-mM NaCl (S2), 5% PEG (D1), and 10% PEG (D2) and interaction of each level of NaCl and PEG at 0, 6, 12, 24, and 48 h after stress induction. The relative signal amplitude was obtained after 28 amplification cycles and normalized to the alpha-tubulin signal obtained after 21 amplification cycles. LSD mean comparison test were performed ($p < 0.05$). Bars are means ± SD

encodes a tonoplast $Na^+/H^+$ Antiporter was rapidly induced by salt stress and constantly remain upregulated from 3 to 24 h [51], which is in line with our findings. Even though the *FaSOS1* gene may play a role in the transport of $Na^+$ ions, we observed that its expression increases in response to drought stress as well, suggesting that this gene may be a component of stress response pathway network that is activated by a common stress signal like ROSs [52] and is involved in maintaining cell homeostasis in stress situations. It also can be concluded that osmotic stress is another key trigger to induce *FaSOS1* expression, since gene expression level increased as osmotic pressure of the solution increased regardless of the type of solute either NaCl or PRG treatment (Table S1). Similar reports on expression of tonoplast $Na^+/H^+$ antiporter published on barley [53] and Moso bamboo [54]. Partially stable induction of *FaSOS1* over time after salt stress is related to the low stability of SOS1 mRNA which is controlled by transcript-specific cis-elements in the coding region of SOS1 [52]. Our results indicate that transcript level of *FaSOS1* and *FaADC1* showed a similar kinetics under drought stress. ABRE-related motifs can be located in the promoters of *ADC1* paralogue [55] and *SOS1* [25, 56], which are highly up-regulated in response to drought stress. These findings suggested that a common signaling factor like ABA may modulate the transcriptional up-regulation of *FaADC1* and *FaSOS1* triggered by drought. Therefore, in response to drought, ABA may act as an upstream regulator of PA biosynthesis and the SOS pathway.

The discovering and identification of ABA biosynthesis genes have evolved significantly in recent years. However, little is known about the molecular mechanisms behind ABA catabolism. Additionally, the catalytic function of the CYP707A1 gene under dual salt and drought stress was unknown, but expression pattern of CYP707A1 provided useful information on the physiological role of this gene under various salt and drought stress treatments. The majority of cytochrome P450 genes have been demonstrated to be affected upon exposure to a single drought, high salinity, or low temperature [58]. Because ABA C-8′-hydroxylase enzymes participate in the breakdown of endogenous ABA, plant *CYP707A1* transcripts can serve as downstream reporters for ABA-related biochemical pathways [57]. Rapid and late reduction of *CYP70A1* transcripts after adding PEG and NaCl respectively, that significantly increases cellular ABA levels [58] may support the response of stomata closure as a reflection of ABA accumulation in leaves in response to stress which is accrued for preventing transpiration at lower water potential [59]. Also, as a result of ABA accumulation it is contributed to the increased activity of CAT and SOD under salt and drought stress as it is reported that exogenous ABA treatment can increase the activity of SOD and CAT in Carpetgrass, Tifdwarf and Tifway bermudagrass [60].

In response to drought stress, *FaCYP707A1* exhibited a gradually ascending kinetic while under salt stress the induction occurred early after 6 h and transcript level slowly decreased under both conditions same as those in single stress except for the sever dual stress (S2 + D2) (Fig. 2). Highly induction of *FaCYP707A1* was observed on *Arabidopsis* 6 h after dehydration [24]. NaCl treatment induced *FaCYP707A1* transcript level after 6 h same as those in *Arabidopsis* [57] and under uniform salt stress on cotton [61]. CYP707A1 expression in response to ABA treatment enhanced gradually over time in wild-type potato plants [62]. Ectopic expression of bZIP transcription factor in potato could drastically increase CYP707A1 transcript level in transgenic lines [62]. The impact of ABA catabolism and signaling in tall fescue survival under salt and drought stress is indicated by the co-regulation of *FaCYP707A1* and

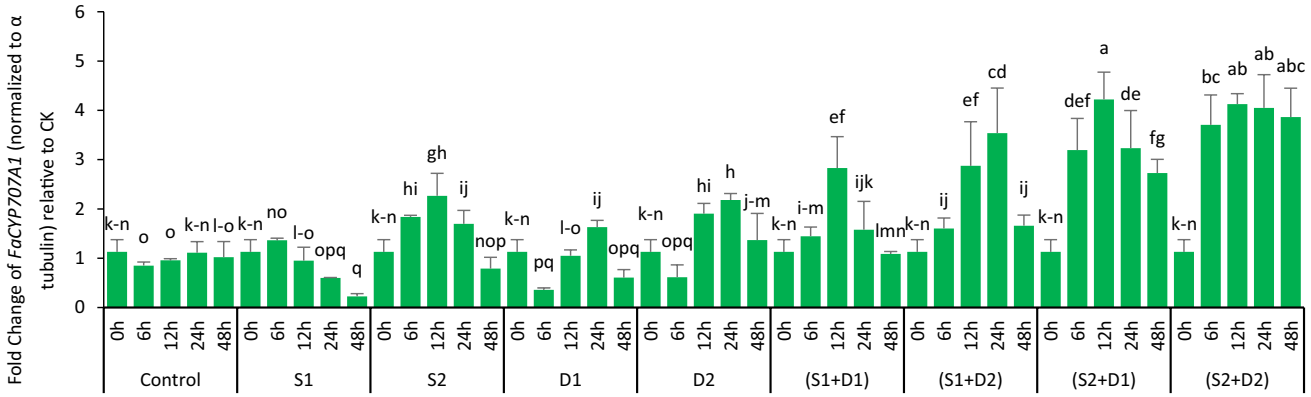


**Fig. 2** RT-PCR analysis of the expression of the *FaCYP707A1* gene in mature tall fescue leaves in half strength Hoagland solution (Control), 200-mM NaCl (S1), 400-mM NaCl (S2), 5% PEG (D1), and 10% PEG (D2) and interaction of each level of NaCl and PEG at 0, 6, 12, 24, and 48 h after stress induction. The relative signal amplitude was obtained after 27 amplification cycles and normalized to the alpha-tubulin signal obtained after 21 amplification cycles. LSD mean comparison test were performed ($p < 0.05$). Bars are means ± SD

*FabZIP69,* respectively. CYP707A and bZIP are two important gene families involved in the catabolism and signaling of ABA, respectively, and several crops obtained tolerance to drought as a result of genetic modification with these genes [24, 62]. However, these results show that *FaCYP70A1* gene is not only regulated by drought but is also up-regulated by severe salt stress.

The basic leucine zipper (bZIP) transcription factors (TFs) are crucial for many biological functions, including plant's response to biotic and abiotic stressors [24, 63]. *FabZIP69* expression levels were up-regulated 6 h after moderate and severe PEG treatment compared with control, and it was approximately 1.5- and 2-fold, respectively, and 12 h after severe drought stress it reaches the peak bud had not changed under moderate drought stress (Fig. 3). Under salinity stress, our qRT-PCR results revealed that *FabZIP69* transcripts were induced 6 h after treatment and upregulated almost 1.5- and 2-fold in response to moderate and severe salt stress, respectively, and then gradually downregulated to the ground level as in the control (Fig. 3). Since the stimulation of transcription factors does not necessitate the synthesis of new proteins and their signaling components are already primed, these genes are expressed early after stress but temporarily [62]. However, *FabZIP69* transcript levels induced under dual stress after 6 h and decreased after 48 h. Unlike to some bZIP family members that are involved in different and unique stress signal pathways [65], *FabZIP69* may be involved in multiple stress responses since it showed induced expression both under NaCl and PEG treatments compared to control plants. Since TFs have the ability to up- or down-regulate the expression of a group of genes that includes particular *cis*-elements in their promoters, we can

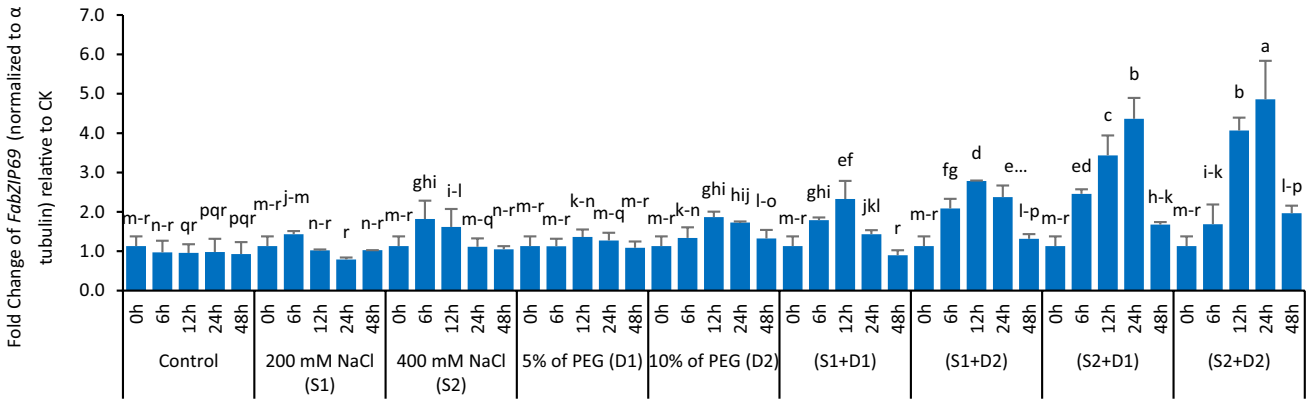


**Fig. 3** RT-PCR analysis of the expression of the *FabZIP69* gene in mature tall fescue leaves in half-strength Hoagland solution (Control), 200-mM NaCl (S1), 400-mM NaCl (S2), 5% PEG (D1), and 10% PEG (D2) and interaction of each level of NaCl and PEG at 0, 6, 12, 24, and 48 h after stress induction. The relative signal amplitude was obtained after 29 amplification cycles and normalized to the alpha-tubulin signal obtained after 21 amplification cycles. LSD mean comparison test were performed ($p < 0.05$). Bars are means ± SD

gain clear insight into the regulation mechanisms of gene expression by looking at the *cis*-regulatory elements in the promoter regions of a gene [24]. Osmotic stress signaling driven on by cold, salinity, and drought stress is modulated by bZIP and MYC and MYB transcription activators that engage with CRT/DRE, ABRE, or MYCRE/MYBRE domains in the promoter of stress response genes [66]. ABRE-binding protein (AREB) and ABRE-binding factor (ABF), which are bZIP transcription factors, can bind to ABRE elements and trigger ABA-dependent gene expression [66]. While SOS1 transporter gene has ABRE (ABA-responsive element) and DRE (dehydration-responsive element) elements in its promoter regions [25], suggesting that *FaSOS1* may also be the target genes of the bZIP-type transcription factors, like *FabZIP69* under stressors. We found a correlation between expression kinetics of *FaSOS1* and *FabZIP69* under stress conditions. Upon high induction of *FabZIP69* 6 h after salt stress and 12h after drought stress, *FaSOS1* expression level was increased. The cis-acting elements ABRE and DRE play a role in the expression of genes in response to stress that are ABA dependent and ABA independent, respectively [67]. The correlation between the expression of tonoplast $Na^+/H^+$ antiporters and bZIP TFs has been observed before, since after overexpression of *OsbZIP71* in rice, *OsNHX1* was up-regulated and also salt and PEG tolerance considerably enhanced [68]. There is also a significant positive correlation between the expression of *FabZIP69* and *FaCYP70A1* supporting the idea of regulating *FaCYP70A1* gene with *FabZIP69* transcription factor since in *Arabidopsis*, ZIP transcription factors directly bind to the cis elements in promoter regions of CYP707A1 and CYP707A3 to regulate ABA levels upon dehydration [57]. Moreover, six ABRE domains were detected in the upstream region of bHLH122 gene which is an up-stream regulatory genes of CYP70A3 in *Arabidopsis* [57] that also explain the high correlation of these genes. The *FabZIP69* gene is associated with salt and drought tolerance in tall fescue due to its ability to initiate a cascade of gene expressions, upregulating ABA-dependent genes, like *FaSOS1* and *FaCYP70A1*. These genes, in turn, activate biochemical and physiological stress responses, including the accumulation of compatible solutes, stomatal closure, and the activation of ROS enzymes [60].

To comprehend how single and dual drought and salt stress can trigger polyamine level responses, the relative expression of the enzyme *FaADC1*, which directly regulates Put biosynthesis in the PAs pathway [23], was measured. Both salt stress levels and types made an obvious increment in *FaADC1* transcript content after exposure. However, we observed an early down-regulation after 6 h of severe salt stress and then cycles of up- and down-regulation in *FaADC1* expression (Fig. 4). Yet, there is evidence that Put levels decline in rice plants under salt stress [22]; and salt treatment did not change the pattern of ADC1 promoter activity [69]. In contrast, early after applying drought stress, *FaADC1* expression increased, and its expression was accelerating throughout the time. The expression of ADC1 also increased during drought stress exposure in rice [70] and also showed similar kinetics to ABA-inducible genes in *Craterostigma* [55]. Moreover, improved osmotic and drought stress tolerance were displayed by the overexpressed ADC of *Arabidopsis* [71]. Under dual stress, *FaADC1* induced as early as 6 h after exposure to stressors and showed a greater amplitude than those in single salt and drought stress (Fig. 4). It appears that *FaADC1* expression is influenced by the kind of stress and the length of exposure which is consistent with Shi et al. report [72]. Early induction of *FaADC1* under osmotic stress might be due to its promoter components, since ABRE or ABRE-related motifs are present in the promoters of ADC paralogue genes [55]. Generally, expression level of *FaADC1* was less than other candidate

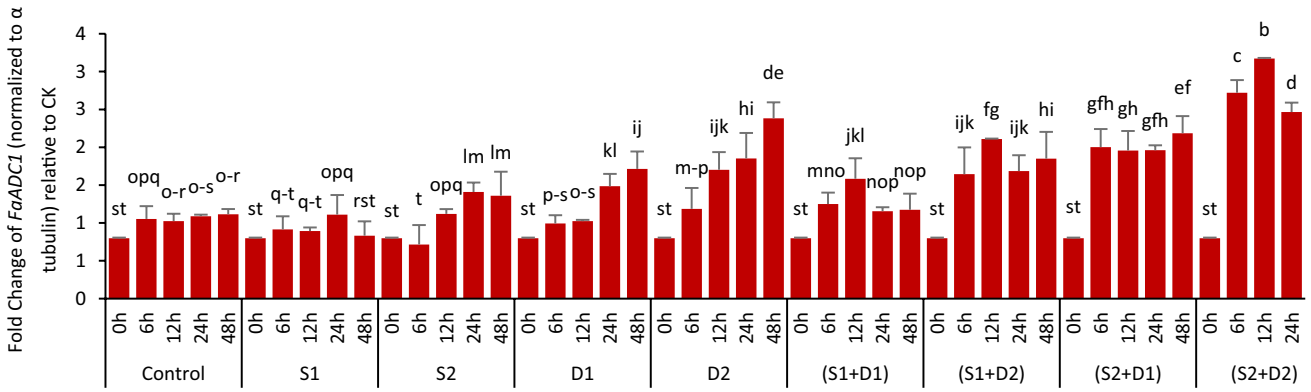


**Fig. 4** RT-PCR analysis of the expression of the *FaADC1* gene in mature tall fescue leaves in half strength Hoagland solution (Control), 200 mM NaCl (S1), 400 mM NaCl (S2), 5% PEG (D1), and 10% PEG (D2) and interaction of each level of NaCl and PEG at 0, 6, 12, 24, and 48 h after stress induction. The relative signal amplitude was obtained after 30 amplification cycles and normalized to the alpha-tubulin signal obtained after 21 amplification cycles. LSD mean comparison test were performed ($p < 0.05$). Bars are means ± SD

genes (< 4-fold), while we observed relatively high expression even on control condition. Additionally, ADC overexpression and stressful conditions caused an osmotic adjustment through Pro and SS buildup caused by PAs [72]. PAs can enhance DNA-binding activity of transcription factors and impose a synergic impact on transcription factor effect [73]. It has been revealed that ADC1 is expressed in all *Arabidopsis* tissues under high-salt concentration (75 mM) [74].

## Morpho-physiological Parameters

Understanding how abiotic disturbances affect the morpho-physiological processes in plants enables us to anticipate the damage under stress condition and deal with it. Our results showed that NaCl and PEG stressors significantly affected RWC, soluble sugar (SS) and proline amino acids content (Pro), total chlorophyll content (Chl), SOD and CAT activities, ion leakage (IL), membrane stability index (MSI), DPPH radical scavenging activity, and $H_2O_2$. Interaction of NaCl and PEG treatment which represents dual stress condition could alter RWC, Chl content, IL, MSI, and H2O2 in leaves significantly ($p \leq 0.01$) (Table S-3).

## RWC and Total Chlorophyll (Chl) Content

The most essential component of the light-harvesting complex (LHCII), which serves as an antenna to gather and direct light energy is Chl [75]. Chl content implies the impacts of stress on plants since stress can make a variety of modifications in Chl biosynthesis and degradation-related genes and consequently, the Chl content [76, 77]. When exposed to drought and salt stress, tall fescue plants showed signs of wilting and the development of chlorosis based on the stress intensity, respectively, in comparison to control plants in Hoagland solution. A significant drop in Chl concentration was caused under dual stress conditions (Fig. 4). Remarkably, under moderate drought and salt stress, the Chl content of the plants had not changed; however, it was decreased by 55.8%, under severe dual stress conditions (D2 + S2) in comparison to the control plants. Reduction of Chl content may be one of the downstream biochemical responses associated with reduction of *FabZIP69* expression after salt and drought treatment (Fig. 5a). Overexpression of a *bZIP* TF genes in soybean improved salt and drought resistance through increasing Chl content and reduction of malondialdehyde [65]. Salt stress caused reduction in chlorophyll content and $CO_2$ assimilation in *Arabidopsis* [68].

Salinity at first step reduces the stomatal conductivity and photosynthesis rate and in this way, Chl content remains undamaged [79]; however severe long-lasting salt stress interrupts Chl synthesis by down-regulating GluTRs and Mg branch involves CHLH, CAO, and POR which are important genes in Chl biosynthesis and increases Chl degradation through ROS production [80, 81]. Salt stress reduces the endogenous amounts of 5-aminolevulinic acid (ALA) through down-regulation of related genes, encoding ALA metabolic enzymes (8082). ALA is a novel plant growth regulator and an intermediate in Chl biosynthesis pathway (8183) and antioxidant machineries [80]. The amounts of total Chl content 48 h after severe drought stress was still high. It has reported that because of upregulation of Chl a/b-binding protein and photosystem II (PSII) oxygen evolving complex PsbQ family protein and down-regulation of a PSII protein D1 under drought stress in rice shoots, these pigment contents did not make significant changes [84]. In contrast to severe dual stress conditions that contributed to welter-ing, plants often remained turgid with a high RWC (> 70%) under moderate and severe single salt and drought stress conditions (Fig. 5b). NaCl at the non-lethal intensity reduces

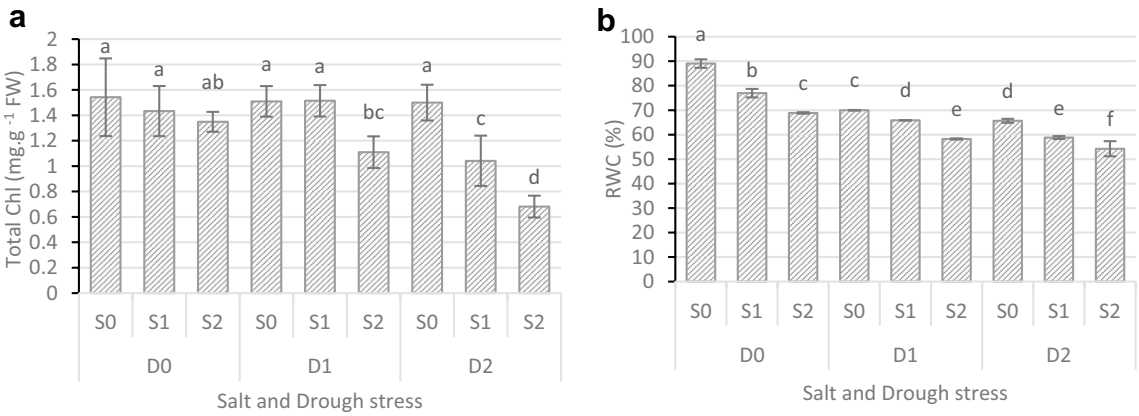


**Fig. 5** Relative water content (RWC) (a) and total chlorophyll (Chl) content (b) of 4-week-old plants after 48 h in half-strength Hoagland solution (S0, D0), 200-mM NaCl (S1), 400-mM NaCl (S2), 5% PEG (D1), and 10% PEG (D2). Data represent means of three replicates. Means not sharing the same letter are statistically significantly different ($p < 0.05$) according to LSD's test. Bars are means ± SD

the RWC and leaf area and in this way, Chl content remains undamaged [85]; however, severe salt stress interrupts Chl synthesis by disrupting ion homeostasis and increase Chl degradation through production reactive oxygen radicals [80, 81].

## Osmotic Adjustments During Salt and Drought Stress in Tall Fescue

Proline (Pro) and soluble sugars (SSs) were further tracked. One significant physiological shift that took place when plants were subjected to stress was the active buildup of solutes inside the cells, which adjusts the osmotic pressure (Fig. 6a, b). In comparison to the control, drought stress increased the amount of SSs in leaf tissue (Fig. 6a). As an adaptation response, tall fescue increases Pro and SSs content under both abiotic stresses (Fig. 6a, b). Tall fescue plants' osmotic adaptations are also highlighted by the enhanced expression of *FaADC1* and *FaSOS1* (Figs. 1, 4). On the other hand, under dual drought and salt stress, the transcript level of the osmoregulatory genes of *FaADC1* and *FaSOS1* elevated by > 3-fold and > 9-fold, respectively, in comparison to control plants. Polyamines mainly control a number of proteins involve in the Calvin cycle, glycolysis, and the gluconeogenesis/glyoxylate cycle, causing the related carbon metabolism to accumulate starch and sucrose [72]. As a result, PAs induce Pro and SSs buildup and reduce osmotic pressure under stressful conditions [86]. In plants under severe NaCl stress treatments, Pro concentration was significantly higher (37.7%) than those in control. Severe water stress enhanced Pro concentration up to 52.8% (from 112 to 171 μg $g^{-1}$ fresh weight). In all stressed treatment subjects, the content of both Pro and SS osmolytes rose during salt and drought stress. Drought and salinity tolerant plants typically have higher concentrations of osmolytes, such as SSs and Pro, as well as non-enzymatic and enzymatic antioxidants [87]. Pro and SSs consider common osmolytes that can relieve osmotic stress brought on by salt and drought stress [86]. Scavenging percentage of DPPH was measured for the antioxidant potential of tall fescue plants. Maximum increase in DPPH activity was observed

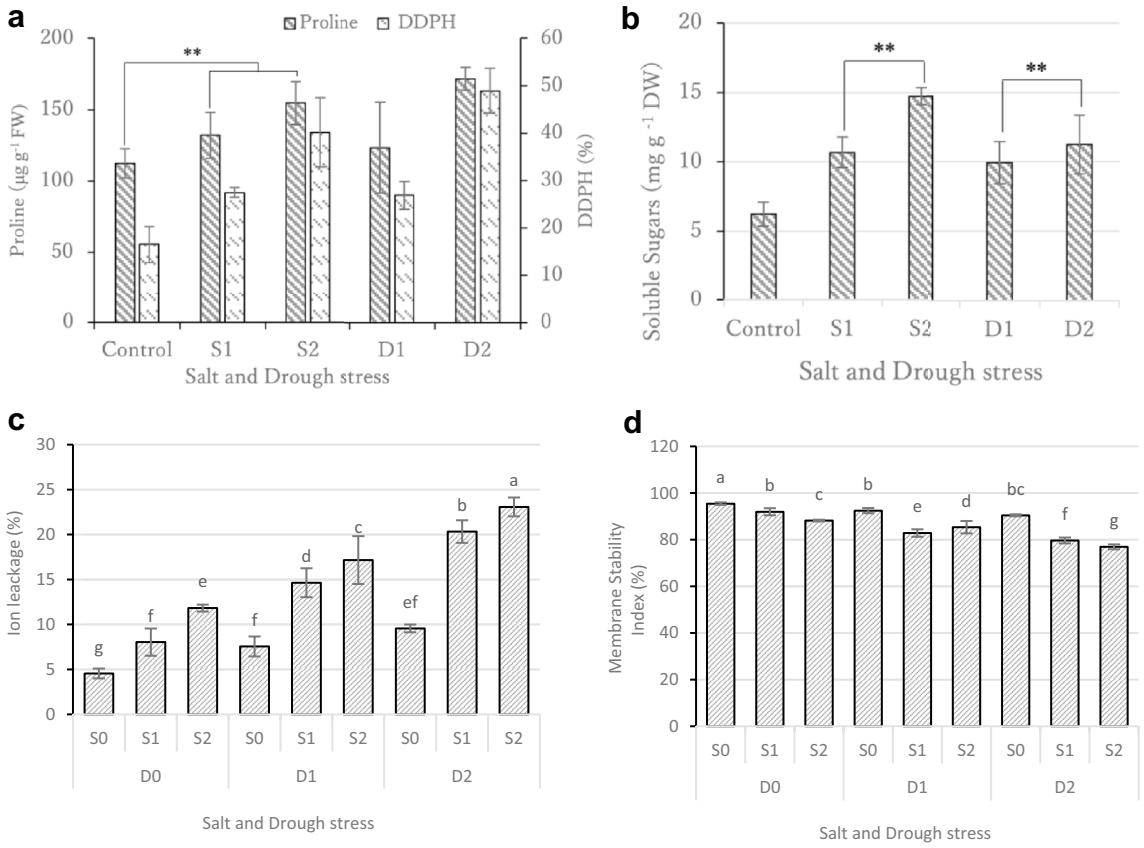


**Fig. 6** Proline content and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging (**a**), soluble sugars (**b**), ion leakage (**c**), and membrane stability index (MSI) (**d**) of 4-week-old plants after 48 h in half-strength Hoagland solution (S0, D0), 200-mM NaCl (S1), 400-mM NaCl (S2), 5% PEG (D1), and 10% PEG (D2). Data represent means of three replicates. Means not sharing the same letter are statistically different ($p < 0.05$) according to LSD's test. Asterisks indicate significant differences at $p < 0.01$ (**). Bars are means ± SD

in severe NaCl- and PEG-treated plants (Fig. 7a) which is contributed to stress tolerance mechanisms.

High concentrations of SSs like trehalose, sucrose, and raffinose maintain membranes, inhibit protein denaturation, and function as ROS scavengers [87]. Since certain polyamines accumulate under various abiotic stressors, they may function as compatible solutes and also osmo-regulators through controlling the osmoregulation-related channels, including aquaporin, $K^+$ channels, and mechanosensitive channels [23]. Upon the application of severe dual stress factors (D2 + S2), the IL increment is drastically detected (40.6%) after 48 h (Fig. 6d); on the other hand, membrane stability reduced by 20% (Fig. 6d). It has been distinguished that this is primarily brought on by the efflux of $K^+$ and what are known as counterions ($Cl^-$, $HPO_4^{2-}$, $NO^{3-}$, citrate$^{3-}$, and malate$^{2-}$), which are launched to balance the efflux of positively charged potassium ions [88].

## Histochemistry DAB Staining, $H_2O_2$ Content and Antioxidant Enzyme Activity

Stained leaves observed under the binocular stereomicroscope showed presence and distribution of hydrogen peroxide ($H_2O_2$) differently between control and treated plants (Fig. 7a). As drought and salt stress increased to 10% PEG and 400-mM NaCl, leaves discolored more compared to those in control in a dose-dependent manner that shows higher $H_2O_2$ content as well (Fig. 7b), as it has been reported that salt and drought stress induce high $H_2O_2$ accumulation which acts as a signaling molecule [86, 89].

There were significant variations in the levels of SOD and CAT enzyme activity after applying salt and drought stress (Fig. 7c). Reactive oxygen species (ROSs), which are byproducts of respiration and photosynthesis, are accumulated by plant mitochondria and chloroplasts during water stress [90]. Plant's feedback to high ROS accumulation is increase in SOD and CAT enzymes' activity. Similar response of CAT and SOD in plants subjected to a NaCl

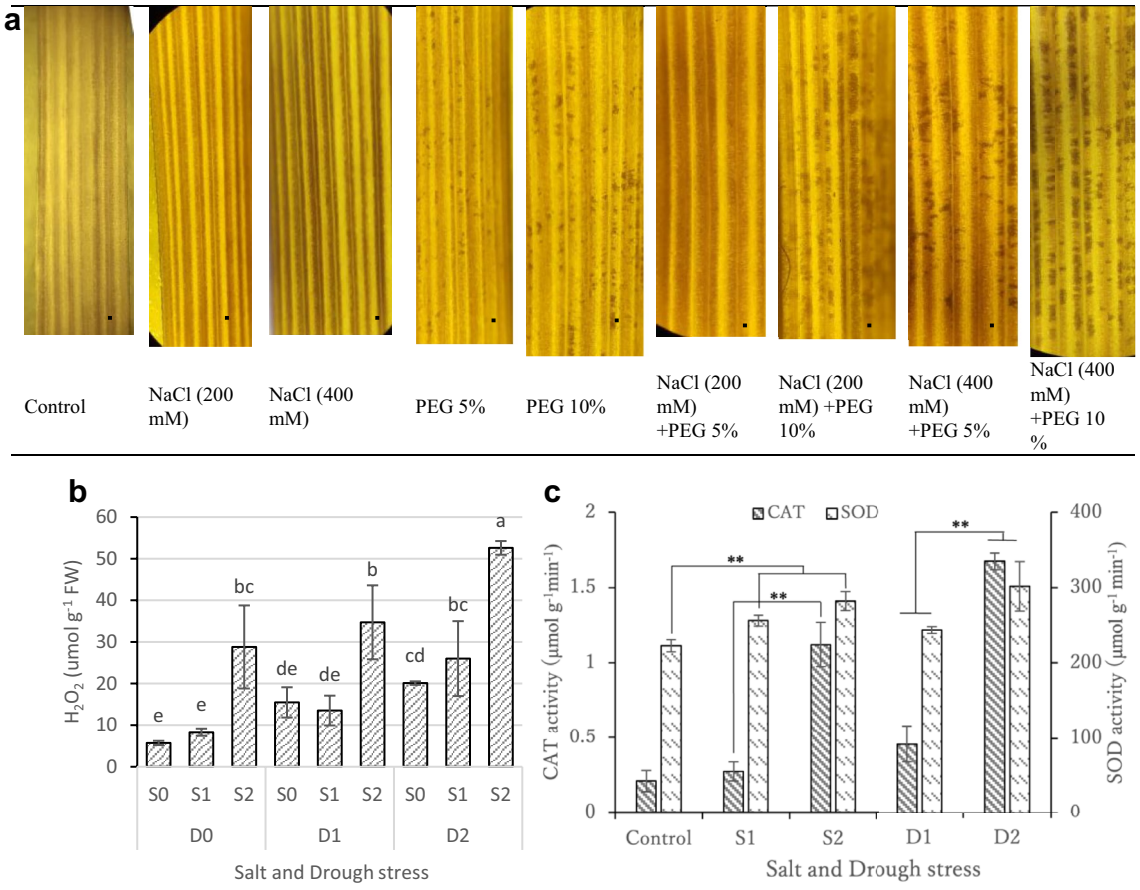


**Fig. 7** Histochemistry DAB staining (**a**), $H_2O_2$ content (**b**), and super oxide dismutase (SOD) and catalase (CAT) (**c**) activity of 4-week-old tall fescue plants after 48 h in half-strength Hoagland solution (S0, D0), 200 mM NaCl (S1), 400 mM NaCl (S2), 5% PEG (D1), 10% PEG (D2). Data represent means of three replicates. Means not sharing the same letter are statistically significantly different ($p < 0.05$). Asterisks indicate significant differences at $p < 0.01$ (**). Bars are means ± SD

and PEG solution may be a sign that the response of these enzymes to salt and drought stress are controlled by similar mechanisms.

### PCA and Correlation Analysis

Recognizing the most effective parameters in stress detection earlier than the adverse effects of stresses become visible can be very useful in large-scale yield management. To investigate the relationship between physiological and morphological parameters with different levels of salt and drought stress, a principal component analysis (PCA), and correlation analysis were performed.

The first two components of PCA pointed out 89.5% of the variance in morpho-physiological parameters. The first component (78.6% variance) was positively correlated with Pro (Pearson's $r > 0.28$), SSs (Pearson's $r > 0.29$), IL (Pearson's $r > 0.28$), and $H_2O_2$ (Pearson's $r > 0.27$) parameters, which corresponded with ion homeostasis and ROS scavenging. On the other hand, the second component (10.9% variance) was correlated positively with total Chl (Pearson's $r > 0.33$), MSI, and SOD which resembled overall stress tolerance (Fig. 8a). The results of PCA may suggest many sensors and signaling pathways for salt and drought stress, for example, SSs, Pro, and ROS (Fig. 8). These results strongly supported the notion that salt and drought responses are mainly mediated by enhancing Pro and SSs and ROS over accumulation especially $H_2O_2$. ROS are signaling initiators that generated during stress conditions relies on the transfer of electrons to oxygen, originating from the electron transport chain in mitochondria and chloroplasts [52]. Recent investigations have shown that SSs and Pro can serve as signaling molecules and their actions are not isolated; instead, they coordinate their actions to improve cell functions to stressors [91, 92].

Through correlation analysis, we found out that there is a highly significant positive correlation between molecular responses Pro, SSs, and IL, with DPPH, SOD and CAT, while they were negatively related to total Chl and RWC (Fig. 8b). It seems that tall fescue is capable in $Na^+$ sequestering and osmotic adjustment under short term abiotic stresses, due to upregulation of *FaSOS1* and *FaADC1* and compatible solutes accumulation that may have modulated toward $Na^+$ efflux and osmotic adjustment [89].

## Conclusion

We studied changes in the expression levels of four stress-inducible genes, *FaSOS1*, *FaADC1*, *FaCYP707*A1 and *FabZIP69*, in tall fescue after exposure to two concentrations of NaCl and PEG over several time points. We found that transcript amount of all genes was increased by applying salt stress after 6-h exposure but their expression kinetics were differently changed throughout the time as the salt concentrations increased. We also found that all of these genes were upregulated after exposure to both concentrations of PEG mostly after 12 h (*FabZIP69* expression was not drastic). In all stressor treatments, *FaCYP707A1* and *FabZIP69* were downregulated after 48-h exposure. All genes exhibited different expression kinetics under single salinity and drought treatments except for *FaSOS1* and *FaADC1*, salt-responsive

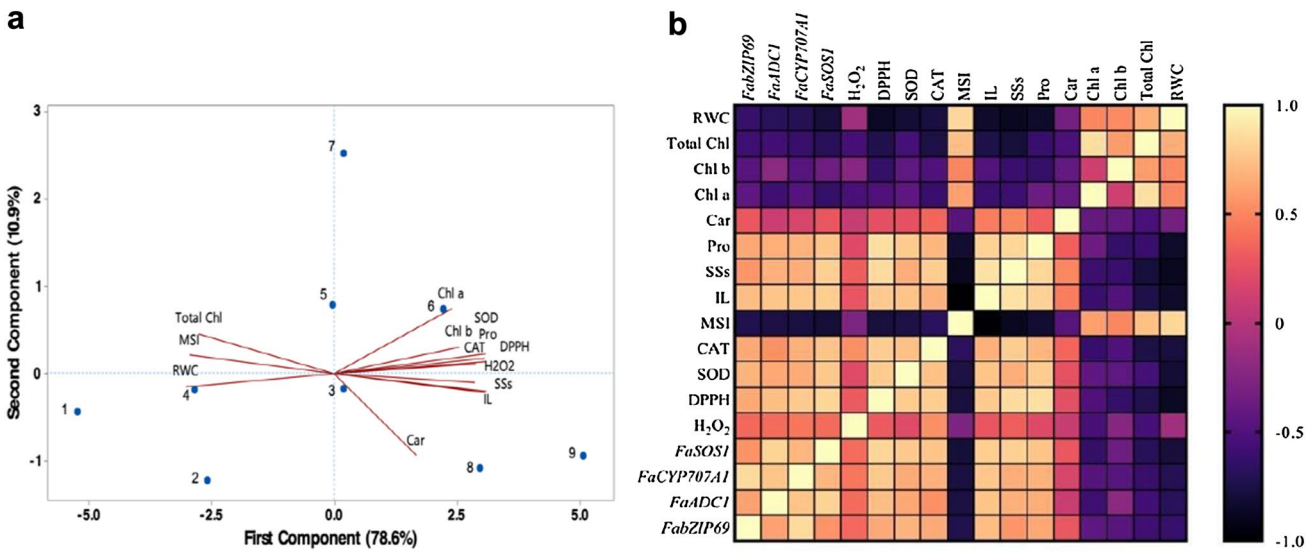


**Fig. 8** Principal component analysis (PCA) (**a**) and heatmap Pearson correlation (**b**) of 4-week-old tall fescue plants after 48-h exposure to NaCl and PEG stressors. Numbers in PCA graph: (1) control, (2) 200-mM NaCl (S1), (3) 400-mM NaCl (S2), (4) 5% PEG (D1), (5) D1 + S1, (6) D1 + S2, (7)10% PEG (D2), (8) D2 + S1, and (9) D2 + S2

gene (*FaSOS1*) was upregulated rapidly (6 h) under salt stress and upregulated lately (24 h) under drought stress and the transcript level remined stable after upregulation, while *FaADC1* showed similar expression kinetic to *FaSOS1* under drought, suggesting their stimulus might be similar like increase in ROS and ABA after stress. *FabZIP69* gene as a transcription factor response to both salt and drought stresses was slightly up- and down-regulated (< 5-fold) during single and double stress conditions. *FaADC1* involved in polyamine synthesis pathway responsive to both salt and drought stresses were up-regulated drastically, early after exposure to both stress conditions. These results suggest that tall fescue may use similar and different mechanisms to handle drought and salt stresses and a combination of mechanisms to handle dual stress. The results of analyzing the biochemical elements gave us a picture of the significance of ROS scavengers, compatible solutes in the single and dual stress in tall fescue plants. Generally, since tested candidate genes belonging to different salt and drought tolerance pathways were upregulated in a similar or different manner, tall fescue may apply both unique and common mechanisms to cope with each of these abiotic stresses.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12033-023-00888-8.

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**Data availability** Data is available by sending request to the first author's email address (elham.esmailpourmoghadam@gmail.com).

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