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ORIGINAL ARTICLE

Differential gene expression reveals candidate genes for osmotic stress response in faba bean (*Vicia faba* L.) involved in different molecular pathways

Ghassen Abid[1] · Mohammed Najib Saidi[2] · Rim Nefissi Ouertani[3] · Yordan Muhovski[4] · Salwa Harzalli Jebara[1] · Emna Ghouili[1] · Khaled Sassi[5] · Jean-Pierre Baudoin[6] · Mohamed El Ayed[7] · Salem Elkahoui[7,8] · Fathi Barhoumi[1] · Moez Jebara[1]

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Abstract

Drought and salinity are the most important environmental constraints affecting faba bean (*Vicia faba* L.) development and crop yield in Tunisia and other Mediterranean countries. Through using different strategies, associating in silico analysis of gene expression and qRT-PCR, this study aims at identifying key genes of faba bean molecular pathways potentially involved in salt and drought response. The impact of these stresses on several physiological and biochemical parameters were investigated in two genotypes (Bachar and Giza 3). To uncover abiotic stress-related genes and better understand the mechanisms of salt and drought stress tolerance in faba bean, a total of 25 faba bean genes were identified through in silico analysis. These genes were associated with important cellular processes such as transcription regulation, signal transport, kinases, phytohormonal signaling, and defense/stress responses. Most of the studied candidates were expressed at various levels in different organs including leaves, roots, flowers, stems, cotyledons, and seeds suggesting a potential role in the growth and development of faba bean plants. Furthermore, qRT-PCR was used to study gene expression profiles in leaves and roots of Bachar and Giza 3 plants under salt and drought stresses, and ABA treatment. The results showed that selected transcripts were differentially expressed under various treatments in both genotypes suggesting their important roles in abiotic stress tolerance responses. The osmotic-responsive genes identified in this study may be considered as potential candidates with a further application as stress selection markers for the creation of faba bean stress-tolerant varieties in various breeding programs.

Keywords In silico analysis · Faba bean · Gene expression · Osmotic stress · qRT-PCR · Salt stress

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✉ Ghassen Abid
gha_abid@yahoo.fr

[1] Laboratory of Legumes, Biotechnology Center of Borj Cedria, University of Tunis El Manar, 901, 2050 Hammam-Lif, Tunisia

[2] Biotechnology and Plant Improvement Laboratory, Centre of Biotechnology of Sfax, Road Sidi Mansour 6 km, PO Box 1177, 3018 Sfax, Tunisia

[3] Laboratory of Plant Molecular Physiology, Biotechnology Center of Borj Cedria, University of Tunis El Manar, 901, 2050 Hammam-Lif, Tunisia

[4] Department of Life Sciences, Walloon Agricultural Research Centre, Chaussée de Charleroi, 234, 5030 Gembloux, Belgium

[5] Laboratory of Agronomy, National Agronomy Institute of Tunisia (INAT), University of Carthage, Avenue Charles Nicolle, 43, 1082 Tunis-Mahrajène, Tunisia

[6] Laboratory of Tropical Agroecology and Horticulture, University of Liège, Gembloux Agro-Biotech, Passage des Déportés, 2-B-5030, Gembloux, Belgium

[7] Laboratory of Bioactive Substances, Biotechnology Center of Borj Cedria, University of Tunis El Manar, 901, 2050 Hammam-Lif, Tunisia

[8] Department of Biology, College of Science, University of Ha'il, P. O. Box 2440, 81451 Hail, Kingdom of Saudi Arabia

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Introduction

Faba bean (*Vicia faba* L.), a legume family member, is one of the most major feed and food legumes ranking fourth worldwide after lentil (*Lens culinaris* Medik.), chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) (http://faostat.fao.org). In Asia, Africa and several developing countries, faba bean is a valuable crop which is a rich source of dietary protein used for human consumption, animal nutrition, and farming systems. In the Mediterranean region, especially countries in North Africa including Tunisia, field faba bean is the most produced grain legume. In 2018, the national production yield was 64,508 tons cultivated on a surface of 54,907 hectares (FAOSTAT 2018). Indeed, Tunisia's average faba bean yield is generally low, at about 1.12 t/ha compared to the worldwide one (2.26 t/ha) (FAOSTAT 2018). Moreover, the yield and production of faba beans in Tunisia, are dependent on seasonal conditions, characterized by wide fluctuations and thus vary significantly from year to year. This unstable productivity is apparently due to a lack of adequate genetic resources. Currently, the crop is cultivated in semi-arid areas where receive average annual precipitation of more than 400 mm per year. Several studies found that drought at various stages of plant development as well as heat during the reproductive growth and pod filling stages are the major problem of this legume (De Costa et al. 1997; Alghamdi 2007; Al-Suhaibani 2009). Furthermore, several studies have shown that faba bean is more susceptible to water deficit compared to common bean, pea, and chickpea (Amede and Schubert 2003). Similar results were also reported by Khan et al. (2010) and Torres et al. (2010). These authors revealed that faba bean is more susceptible to water deficit than other grain legumes mainly due to its relatively shallow rooted and its disability to mediating osmotic adjustment under water-limited conditions. Ouji et al. (2016) mentioned that water deficit is the major constraint to pulse crops production in cultivated fields of semi-arid environments of Tunisia characterized by terminal drought stress.

Drought stress is a major environmental stress that affects numerous metabolic, physiological, and biochemical processes, limiting plant growth and crop yield worldwide (Pandey et al. 2017). Ghassemi-Golezani et al. (2009) revealed that water deficit reduced plant growth, grain filling, grain weight, grains per plant and consequently considerably reduced grain yield in faba bean cultivars. Similar data were reported by Abid et al. (2017) who showed that water limitation reduced growth (leaf area, root fresh weight, shoot fresh and plant height) and physiological (net CO_2 assimilation rate and relative water content) parameters, the activity of antioxidant enzymes and reduced mean grain yield in 11 studied faba bean genotypes. Plant responses to water deficit are complex, involving multiple levels of regulation (Sourour et al. 2017). It is worthy to mention that understanding these responses will help the screening for genotypes tolerant to water-limited conditions. To cope with environmental stresses, plant activates multiple responses involving cascades of gene networks and molecular signaling controlling abiotic stress response in plants including biosynthesis of plant hormones and activation of process of reactive oxygen species (ROS) detoxification in plant cells (Krannich et al. 2015).

High-throughput next-generation sequencing (NGS) technologies including RNA-Seq (RNA sequencing) (Song et al. 2017), DNA microarray (Khan et al. 2017), and in silico analysis (Muthuramalingam et al. 2017) help in the recognition of drought stress-related genes and in enlightening of metabolic pathways affected by drought stress and consequently leading to an acceleration in faba bean crop improvement through genetic and genomic tools.

In silico analysis approach has been successfully used in many species like *Glycine max* (soybean), *Medicago truncatula* (barrel medic), *Cajanus cajan* (pigeon pea), *Phaseolus vulgaris* (common bean) and *Oryza sativa* (rice) (Soares-Cavalcanti et al. 2012; Zhang et al. 2013; Sinha et al. 2016; Büyük and Aras 2017; Muthuramalingam et al. 2017, respectively) for the analysis of global patterns of gene expression in response to various abiotic stress. Indeed, identification and molecular characterization of osmotic stress-responsive genes known from the model legume *Medicago truncatula* in faba bean using in silico approach could contribute to a better comprehension of the early mechanisms at the molecular level and pathways involved in osmotic stress response and adaptation in this leguminous crop.

Drought is a potential constraint which alters plant metabolism and consequently leads to decreases of plant growth and yields by influencing various biological processes of plants like photosynthesis, photosynthetic pigments biosynthesis, plant water relations, hormone biosynthesis, ion uptake and translocation, nutrient metabolism and respiration (Nadeem et al. 2019). During the processes, generating signals in various cell organelles caused by perturbations due to stress were perceived. These signals activate the differential expression of various proteins which were found to be involved in signal transduction including transcription factors comprising members of ABA signaling pathways and other regulatory proteins (transcription factors like ZFP, bZIP, WRKY and NAC), protein kinases and/or phosphatases, redox regulatory proteins, drought-related, proteins proteins related to sugar metabolism and osmotic adjustment leading to plant protection against drought stress (Li et al. 2016; Yang et al. 2019). The main objective of this study is to investigate molecular pathways contributing to osmotic

stress responses in faba bean. For this aim, in silico and transcriptomic analysis were employed to identify and study the expression of candidate genes by qRT-PCR in two faba bean genotypes with contrasting osmotic stress response. The findings are valuable and can serve as a basis for developing of faba bean varieties resistant to osmotic stress.

Materials and methods

Plant material

Two genotypes (Giza 3 and Bachar) were used in the present study for comparative expression analysis to identify putative osmotic stress-response genes in faba bean (*Vicia faba* L. var. *minor*). Giza 3 genotype acknowledged susceptible to drought stress (Abdellatif et al. 2012) was obtained from ICARDA (Aleppo, Syrian Arab Republic) and the one at Bachar, tolerant to drought stress (Maazaoui et al. 2016) from Regional Field Crop Research Center of Beja (CRRGC, Tunisia). The present work was conducted during the period of 2017–2018 in a glasshouse compartment under controlled environmental conditions with 23 ± 2 °C temperature, 55/65% relative humidity, 270 µmol of photons/m^2/s PAR and 16/8 h photoperiod day/night regime. Seeds were surface sterilized by immersing in sodium hypochlorite (NaOCl at 5%) for 5 min and then washed in sterile distilled water several times. After, seeds were soaking for 12 h in distilled water before placed on perlite for germination as reported by Mhamdi et al. (1999). Two weeks old, seedlings were pulled and the roots were washed 5 times with distilled water. Afterwards, a total of 90 seedlings from each cultivar were grown hydroponically in a 40 × 17 × 10 cm (length, width and height, respectively) nutrient reservoir containing 5 L of Hoagland solution (Hoagland and Arnon 1950) in a randomized complete block design with three replicates. The experimental boxes were continuously aerated with aeration tubes and covered with black papers to avoid the algal growth.

NaCl (sodium chloride), ABA (abscisic acid) and PEG (polyethylene glycol) treatment

Salinity and osmotic stresses were applied by using NaCl (200 mM) and PEG-6000 (21%), respectively. After one week of the establishment of the seedlings in Hoagland solution, the nutrient medium was replaced with Hoagland solution (5L/box) containing PEG-6000 and NaCl. Moreover, non-treated faba bean seedlings were used as a control. Leaves and roots were collected at 0 (control), 3, 6, 12, 24 and 48 h time points after each treatment. Besides, ABA treatment (100 µM) was carried out by adding it into the Hoagland culture solution and spraying on the leaves of the 3-week-old seedlings. Leaves samples were harvested at 0, 1, 3, 6 and 12 h after treatment and all control plants were treated with sterile distilled water. Plant materials collected at each sampling time were frozen in liquid nitrogen and stored at − 80 °C until further use. Each treatment contained three biological replications and repeated three times.

Relative water content (RWC)

The RWC was evaluated during 48 h of stress treatments as described by Downey and Miller (1971). To obtain the fresh mass (FM), five fully expanded leaves were randomly taken from the control or osmotic/salt-treated groups of each genotype at the same time point during the stress period and weighted. Then, samples were instantly floated on distilled water in the dark for 24 h at 4 °C to estimate the turgid weight (TW). Finally, samples were dried at 70 ± 2 °C for 3 days and weighed (dry mass, DM).

RWC was determined according to the following equation:

$$\mathrm{RWC}\ (\%) = \left[(\mathrm{FM}-\mathrm{DM})/(\mathrm{TW}-\mathrm{DM})\right] \times 100.$$

Malondialdehyde content (MDA)

Lipid peroxidation level was measured by determining malondialdehyde (MDA) content in plant tissues according to Hodges et al. (1999). Samples (0.5 g) of leaf tissues were homogenized in 10 ml of 0.1% (w/v) TCA (trichloroacetic acid). The mixture was centrifuged at 12,000 rpm for 15 min. A 2 ml aliquot from the supernatant was added to 2 ml of 10% TCA contained 0.5% TBA (thiobarbituric acid). Samples were placed in a water bath at 100 °C for 30 min and centrifuged at 12,000 rpm for 15 min after cooled by incubation on ice. Then the absorbance was measured at 440, 532 and 600 nm in the spectrophotometer (Spectro UV–Vis Dual Beam PC, UV-S-2007; LABOMED, INC.). An extinction coefficient of 155/(mM/cm) was used to determine MDA content (Heath and Packer 1968).

Hydrogen peroxide content (H_2O_2)

H_2O_2 level in tissues was estimated colorimetrically following the method of Mukherjee and Choudhuri (1983). Briefly, 0.5 g of frozen leaf powder was mixed with 3 ml of refrigerated acetone (10%) and centrifuged at 12,000 rpm for 15 min. A 2 ml of extracted solution was homogenized with 1 ml of titanium sulfate (1%) and 0.5 ml of 30% ammonium hydroxide solution. After the precipitate was formed, the homogenate was centrifuged at 4 °C for 10 min at 12,000 rpm. The resulting precipitate was dissolved in 10 ml of 2 M H_2SO_4 after washing three times with acetone and

the absorbance was recorded at 415 nm. The H_2O_2 content was calculated by referring to a H_2O_2 standard curve. Each measurement was repeated at least three times.

Free proline content

Proline was estimated following the method of Bates et al. (1973). A 0.1 g of dry leaf tissues were extracted in 10 ml of 3% (w/v) sulfosalicylic acid over-night. The extract was centrifuged for 10 min at 5000 rpm. Two ml of extracts were homogenized with an equal volume of fresh ninhydrin solution and glacial acetic acid before heading for 1 h in boiling water. Subsequently, free proline was extracted by adding 4 ml toluene. Proline content was estimated by read absorbance at 520 nm using L-proline as a standard. The analyses were performed in triplicate.

Total soluble sugars content

The soluble sugar was determined spectrophotometrically according to the method of Dubois et al. (1956). A 0.1 g of dry leaf tissues was extracted by heating for 15 min in a 95 °C water bath by adding 5 ml of 95% ethanol (v/v) solution. After centrifugation at 3000 rpm for 10 min, the supernatants of the alcoholic extracts were kept in the refrigerator at 4 °C for the determination of total soluble sugars content. Indeed, 200 µl of extract were homogenized with 1.8 ml of sterile deionized water and the samples were rigorously mixed. After adding 2 ml of phenol (5%) and 10 ml of sulphuric acid to each sample, the homogenate was kept at 25 °C for 1 h. Total soluble sugar contents were recorded at 490 nm using glucose as a sugar standard.

Database searches

Li et al. (2009) performed a DNA microarray in salt-stressed roots of *Medicago truncatula* treated with 180 mM NaCl for 6, 24, and 48 h in order to identify salt stress-response genes and regulatory pathways associated with salt stress tolerance. Data of the gene expression quantification was deposited at Gene Expression Omnibus (GEO) under series number GSE13921. According to these authors, the log2 fold change [logFC] values were used to identify the differentially expressed genes (DEGs) in respective stress. Indeed, up-regulated or down-regulated genes were defined that its fold change was positively higher or negatively lower than two times of the standard deviation in any of the three time points. Publicly available EST database of faba bean were downloaded from dbEST of NCBI. The identified *Medicago truncatula* sequences were used for data mining the NCBI in search for faba bean homologues using an *E* value cutoff of 1×10^{-4} (Table 1). BLAST algorithm and NCBI databases (EST, nucleotide and protein) were used for homology searches.

Extraction of total RNA and cDNA synthesis

Total RNA was extracted from leaves and roots tissue according to the CTAB method as reported previously by Chang et al. (1993). The quality and concentration of total RNA was measured spectrophotmetrically using UV-2700 (Shimadzu, Tokyo, Japan) by determining the ratio of absorbance at 260, 280 and 230 nm and its integrity were checked by agarose gel electrophoresis (1.2%). The remaining genomic DNA traces from RNA samples was removed by treatment with 1 µl of DNase I (Biomatik; Wilmington, Delaware, USA); then 2 µg were used for first-strand cDNA synthesis using 200 U MMLV reverse transcriptase (Biomatik; Wilmington, Delaware, USA) according to the manufacturers' instructions. The cDNA products were stored at − 20 °C until used for PCR analysis.

Semi-quantitative RT-PCR

Organ-specific gene expression in roots, leaves, stems, and cotyledons was determined in Bachar 3-week-old seedlings while in flowers (0 days after pollination) at 8-week-old Bachar plants. In seeds gene expression was measured 9 days after pollination. Total RNA was extracted according to the CTAB method and first-strand cDNA synthesis was carried out as described over. Primers were designed using Primer3 program (Rozen and Skaletsky, 2000). PCR reactions were performed in a 20 µl final volume as follows: 95 °C for 5 min; 25–35 cycles of 95 °C for 30 s, 55–60 °C for 30 s and 72 °C for 30 s. A final elongation step was performed at 72 °C for 5 min. Faba bean *VfEF1α* was used as an internal standard for data normalization. Reactions which replicated three times were amplified on a DNA thermal cycler (Applied Biosystems® 2720, Foster City, CA, USA). An aliquot of 15 µl of obtained products was electrophoresed on a 1.2% agarose gels and DNA bands were stained with ethidium bromide before exposed at 254 nm for visualizing.

Quantitative RT-PCR (qRT-PCR)

For qRT-PCR, reactions were run in a 7300 Real-Time PCR thermocycler (Applied Biosystems, Foster City, USA) and were performed in a final volume of 25 µl containing 200 µM primers (Table 2), 12.5 µl Maxima SYBR Green Master Mix, 2 µl cDNA and 9 µl H_2O. The amplifications of all genes were technically replicated 3 times using the following program: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 1 min. Melting curve analysis was performed by heating from 60 to 95 °C, 0.5 °C per 5 s increments. Results were analyzed by StepOne™ Software v2.2.2 (Applied Biosystems, USA).

Table 1 Summary of faba bean (*Vicia faba*) selected genes showing homology with osmotic stress related *Medicago truncatula* genes

Medicago truncatula accession number	Name	Annotation	*Vicia faba* GenBank accession ID	Nucleic acid identity	*E* value
XM_003626566	COMT	Caffeic acid *O*-methyltransferase	JR969443	(699/833) 84%	0.0
XM_003623148	GST	Glutathione *S*-transferase	JR964463	(636/793) 80%	0.0
XM_013605137	DELLA	DELLA domain GRAS family transcription factor GAI (Gibberellic Acid Insensitive)	GARZ01002519	(1349/1678) 80%	0.0
XM_013601862	Nod19	Stress up-regulated Nodulin 19 protein	JR965978	(529/610) 87%	0.0
XM_013599226	LEA3	Late embryogenesis abundant protein, group 3	GARZ01016242	(510/685) 74%	1.00E−120
XM_013609300	NAC	NAC transcription factor	JR969093	(336/393) 85%	1.00E−124
XM_003615931	POX	Peroxidase family protein	GARZ01003046	(586/657) 89%	0.0
XM_013613044	ERF	Ethylene response factor	EU543659	(435/532) 82%	7.00E−143
XM_013613277	MYB-related	MYB transcription factor	JR965237	(684/802) 85%	0.0
KT878752	MATE	Multidrug and Toxic Compound Extrusion (MATE) efflux family protein	GASA01005420	(706/804) 88%	0.0
XM_003608411	LEA6	Late embryogenesis abundant protein, group 6	GASA01006764	(224/282) 79%	2.00E−59
XM_003607126	HMT	Heavy metal transport/detoxification superfamily protein	GARZ01016630	(372/455) 82%	6.00E−117
XM_003615068	PS/TP	Protein serine/threonine phosphatase family	GASA01004694	(605/656) 92%	0.0
XM_003628967	TMEM	Transmembrane protein	JR967913	347/392(89%)	2.00E−140
XM_003614407	ERF-B3	AP2/ERF and B3 domain transcription factor	GARZ01000219	467/578 (81%)	5.00E-150
XM_013605212	TK	Tyrosine kinase family	GASA01012074	(1180/1393) 85%	0.0
XM_003609972	WRKY	WRKY family transcription factor	GARZ01000588	(522/678) 77%	6.00E−150
XM_013601128	LEA	Late embryogenesis abundant protein	GARZ01002376	(525/624) 84%	0.0
XM_003597279	MYB	MYB transcription factor	GASA01006303	(729/889) 82%	0.0
XM_003610053	AP2LP	AP2-like ethylene-responsive transcription factor	GASA01011648	(411/444) 93%	0.0
XM_003616684	ERD	Early response to dehydration-protein	GASA01000181	(553/717) 77%	2.00E−142
XM_003606823	RD22	Dehydration-responsive RD22-like protein	JR966180	274/299 (92%)	2.00E−115
XM_013597564	DHN	Dehydrin	GASA01012523	(185/214) 86%	6.00E−64
XM_003624197	UDP	UDP-glucosyltransferase family protein	GARZ01007510	872/994 (88%)	0.0
XM_003595546	CIPK	CBL-interacting kinase	JR967621	(896/1023) 88%	0.0

Relative gene expression analysis was accomplished with $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). The R package was used to generate heatmaps of differential gene expression.

Statistical analyses

Data were obtained from at least three replicates and represent mean ± standard deviation (SD). One-way analysis ($P < 0.001$) was applied to determine the significance of the results between different treatments and then Turkey's multiple comparison tests at 5% ($P < 0.05$) were performed. ANOVA analysis was conducted using statistical software SPSS 13.0.

Results

Physiological analyses

Characterization of the genotypes differing in their response to osmotic and salt stress was performed by measurement of physiological parameters such as RWC. The RWC was not significantly different between studied genotypes under control condition (Fig. 1a, b). However, underwater stress (3 h, 6 h and 12 h), Giza 3 exhibited slightly, not significant, lower RWC than Bachar. However,

Table 2 Primers used for RT-PCR analysis

Gene name	GenBank accession ID	Forward and reverse primer 5′–3′	Tm (°C)
VfCOMT	JR969443	Forward: 5′-TTTCCTCCTGGATTTTGTGC-3′ Reverse: 5′-GAAAGGTTATTGCGGTGGAA-3′	57
VfGST	JR964463	Forward: 5′-CTTACCGCACCCAAAAACTT-3′ Reverse: 5′-CCAATCTTGCCTTCAGATCC-3′	56
VfDELLA	GARZ01002519	Forward: 5′-AAGACGAACCCCAGTTTCCT-′' Reverse: 5′-GGGAGAGTGAAACGGAATCA-3′	58
VfNod19	JR965978	Forward: 5′-CCCATATTCCGGATCCTTTT-3′ Reverse: 5′-CACTCCGTGCACCCTAACTT-3′	60
VfLEA3	GARZ01016242	Forward: 5′-ATCATCATTCCCCATTCCAA-3′ Reverse: 5′-CGGGCTTCTAGAGACAGCAG-3′	57
VfNAC	JR969093	Forward: 5′-ATGCTGCATCGTTCTCAGTG-3′ Reverse: 5′-TGATTGGGTTCTGTGTCGAA-3′	56
VfPOX	GARZ01003046	Forward: 5′-TTATTGCGTTTGCATTTCCA-3′ Reverse: 5′-TTCTCACCACGGAAGGTAGG-3′	55
VfERF	EU543659	Forward: 5′-TGCTGCTTTTCATTTTCGTG-3′ Reverse: 5′-AGGCGCTGTAAGAGGCATAG-3′	59
VfMYB-related	JR965237	Forward: 5′-CGTGCTCTCCCAAAACTAGC-3′ Reverse: 5′-AATTGCTGACCACCTGTTCC-3′	57
VfMATE	GASA01005420	Forward: 5′-GCCTTGAGCAGCAAGTAACC-3′ Reverse: 5′-TCTTAGAGGAAGCGCGAGAC-3′	57
VfLEA6	GASA01006764	Forward: 5′-CCAACAAACGAAGCAGAACA-3′ Reverse: 5′-CCGTTGGTTCTTGATGACCT-3′	59
VfHMT	GARZ01016630	Forward: 5′-AATTCGTTTCACGACCTTGG-3′ Reverse: 5′-AGGGGTGACACAAGTGGAAG-3′'	57
VfPS/TP	GASA01004694	Forward: 5′-TTGCAGTTACAGCCGTTGAA-3′ Reverse: 5′-AGGATGGATTTTGGGAGGAC-3′	60
VfTMEM	JR967913	Forward: 5′-GCTGAGCCAGAATATGTTCCTC-3′ Reverse: 5′-TGATTGCTGCAACAAGATGAG-3′	58
VfERF-B3	GARZ01000219	Forward: 5′-ATGTTGACGAATCGGTTTCC-3′ Reverse: 5′-ATTCGAAGTCGGAGATCGTG-3′	58
VfTK	GASA01012074	Forward: 5′-TGTTCTGCCGACAAGCATAG-3′ Reverse: 5′-TTTAGTCGAGTGGGCCAAAC-3′	57
VfWRKY	GARZ01000588	Forward: 5′-CCGCTGTTTGCAGTTATTGA-3′ Reverse: 5′-TCATTCATTTCGGTCCACAA-3′	58
VfLEA	GARZ01002376	Forward: 5′-TGACCAGAAGCCAGTGTGAG-3′ Reverse: 5′-CGGGAGTACCAACGGATATG-3′	56
VfMYB	GASA01006303	Forward: 5′-TCCGTTCGACCAGGTAACTT-3′ Reverse: 5′-ATCCTGGTCTCAAACGTGGT-3′	56
VfAP2LP	GASA01011648	Forward: 5′-CGGCATAGATGGACAGGAAG-3′ Reverse: 5′-CCCAGTACTTGAGAGCAGCA-3′	59
VfERD	GASA01000181	Forward: 5′-CAACCACAACAATGGAGACG-3′ Reverse: 5′-CCACCAGTCATCGGAGAAAT-3′	58
VfRD22	JR966180	Forward: 5′-AGAGTTTCCCTTGTCGGTGA-3′ Reverse: 5′-TGCCCCCAATGAGAAGTATC-3′	56
VfDHN	GASA01012523	Forward: 5′-CAGATGAAACAAACTACTCAAAC-3′ Reverse: 5′-AAGCTTCCTGGTACTGGAGGA-3′	55
VfUDP	GARZ01007510	Forward: 5′-TGTGGCCCAGTTCTCTAACA-3′ Reverse: 5′-TTGGTATGGAAGGCCCTATG-3′	56
VfCIPK	JR967621	Forward: 5′-GATCGGCTTTCCAAACGATA-3′ Reverse: 5′-AGGTGGCCCCTTCAATTTAC-3′	58
VfELF1A	AJ222579	Forward: 5′-GTGAAGCCCGGTATGCTTGT-3′ Reverse: 5′-CTTGAGATCCTTGACTGCAACATT-3′	58

at 24 h and 48 h, Giza 3 showed a slightly higher but not significant RWC value compared to Bachar. A similar pattern was obtained under salt stress at 3 and 6 h, whereas at 12, 24 and 48 h Bachar showed higher and significant RWC value compared to Giza 3. In general, under salt stress conditions Giza 3 showed lower RWC value than Bachar. Thus, under equivalent osmotic potential, the effects of NaCl were more harmful than PEG on RWC of Bachar and Giza 3.

Proline accumulation was used as a drought-tolerance selection criterion. In this study, leaves of both studied genotypes showed an increase of proline accumulation under osmotic and salt stress conditions (Fig. 1c, d). Under osmotic stress, the increase varied from 31-fold at 48 h, 1.90-fold at 3 h, 1.60-fold at 6 h and 0.80-fold at 12 h for Bachar and only 5.35-fold at 48 h for Giza 3. Thus, 48 h after osmotic stress induction, Bachar registered the biggest proline content, being followed by Giza 3, and the lowest content was registered at 24 h in both studied genotypes. Moreover, leaves of Bachar showed a high proline content under salt stress during the experimental period. The increase varied from 4.50-fold at 48 h, threefold at 3 h, 1.90-fold at 6 h, 1.05-fold at 12 h and 0.75-fold at 24 h. In Giza 3 an increase by 0.60-fold only at 48 h was observed. Overall, the behavior of Bachar and Giza 3 under osmotic and salt stress showed genotypic variability in the accumulation of proline.

In the present study, data in Fig. 1e, f showed that the initial level of soluble sugar of Bachar and Giza 3 plants was not significantly different under control condition. On the other hand, total soluble sugars content in Bachar and Giza 3 was significantly higher under osmotic and salt stress application compared to the respective controls. Moreover, Bachar had the highest soluble sugars content during osmotic stress; while the lowest soluble sugars content was observed in Giza 3 (Fig. 1e). In Bachar, soluble sugars content increased after 3, 6, 12, 24 and 48 h, approximately up to 9, 9.6, 11, 10.5 and 5.8 folds, respectively. Similar pattern in soluble sugars accumulation was also observed under salt stress and control (Fig. 1f). Osmotic and salinity treatments significantly increased soluble sugars concentration in Bachar compared to Giza 3.

Data shown in Fig. 1g, h revealed that under osmotic and salt pressure, the H_2O_2 content increased significantly in both genotypes compared to the control plants. The responses of both genotypes to osmotic and salt stress were different. For all time points analyzed, osmotic challenged Giza 3 plants exhibited the highest H_2O_2 content. At 3, 6, 12, 24 and 48 h, the H_2O_2 level increased to about 31%, 44%, 76%, 96% and 138%, respectively in Bachar and to about 52%, 76%, 76%, 110% and 147%, respectively in Giza 3 in comparison to the controls (Fig. 1g). Similarly, following treatment with NaCl, the Bachar and Giza 3 leaves (Fig. 1h) displayed an increase in H_2O_2 concentration at each time point of the time course. Although a higher H_2O_2 induction in Giza 3 leaves prior to the beginning of the NaCl treatment was observed (Fig. 1h), at 3 h after treatment Bachar and Giza 3 revealed a similar H_2O_2 content. However, a greater augmentation in H_2O_2 was observed in Giza 3 compared to Bachar, when subjected to salt stress at 6, 12 and 24 h.

MDA content, as an indicator of lipid peroxidation, was induced during the NaCl and PEG treatments in both studied genotypes (Fig. 1i, j). Giza 3 osmotic stressed plants showed higher levels of MDA than Bachar after 0, 3 and 6 h of treatment. However, no significant changes in MDA concentration were observed between Giza 3 and Bachar after 12, 24 and 48 h of osmotic stress induced by PEG (Fig. 1i).

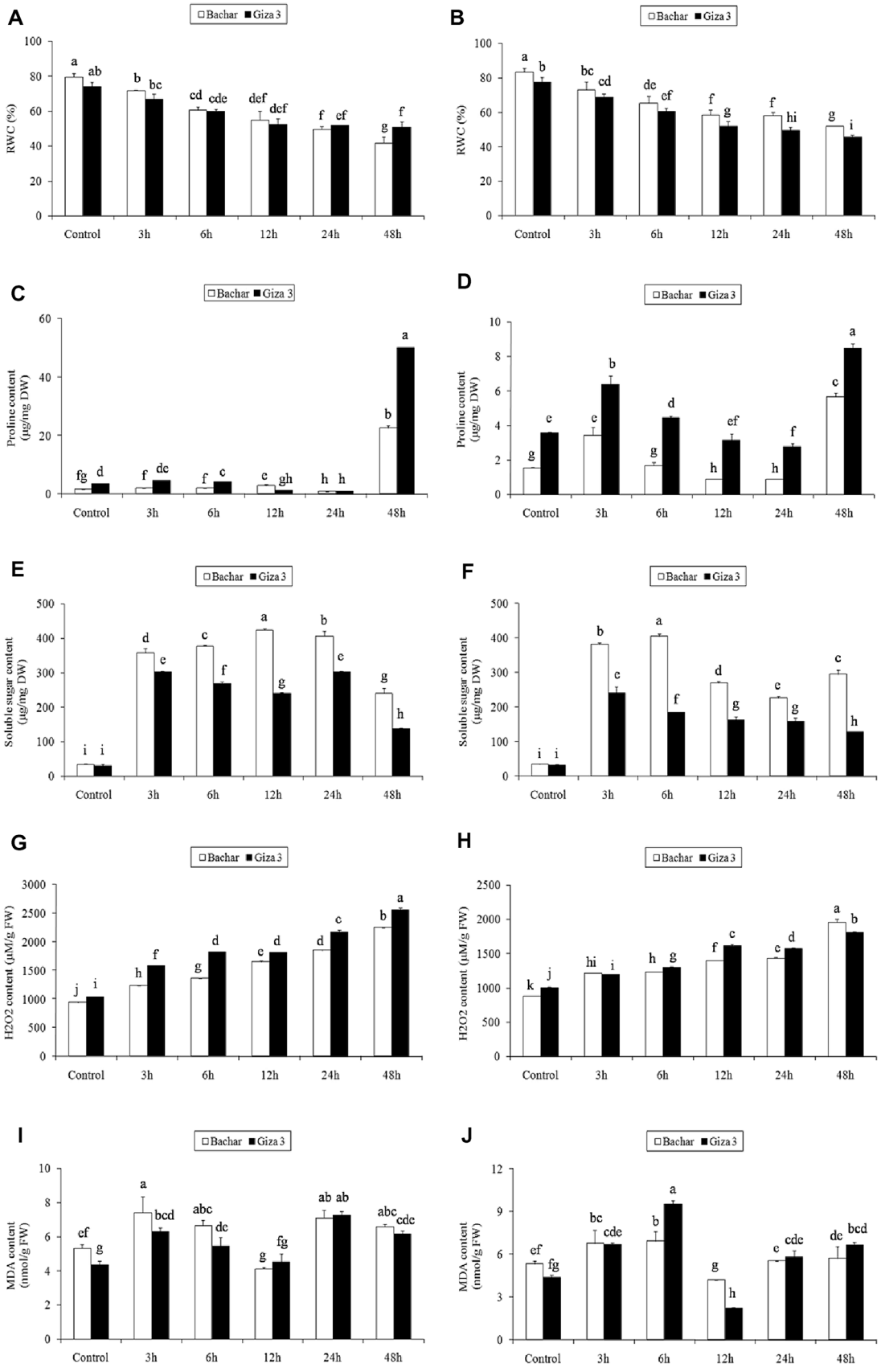
Similar to the changes in H_2O_2 content, after exposure to osmotic stress, MDA increase was significantly higher in Giza 3 than in Bachar. Indeed, MDA accumulation in Giza 3 significantly increased by 40%, 25%, 36% and 23% at 3, 6, 24, and 48 h, respectively, compared to control. NaCl treatment showed changes very similar to PEG which resulted in a significant increase in MDA content at 3, 6, 24 and 48 h. In contrast, there was a remarkable decrease in MDA accumulation at 12 h in both genotypes (Fig. 1j). Compared to control plants, MDA accumulation significantly increased by 53%, 118%, 33% and 51% at 3, 6, 24 and 48 h respectively in Giza 3; while MDA accumulation significantly increased by 53%, 30%, 5% and 6%, at 3, 6, 24 and 48 h respectively in Bachar.

Expression profiles of selected genes in different faba bean tissues

To highlight the roles of the 25 selected osmotic stress-responsive genes (Table 1) in plant development, PCR was conducted to determine their tissue specificity patterns in leaves, cotyledons, roots, stems, flowers and seeds (Fig. 2). Faba bean elongation factor 1-alpha (*VfELF1A*) was used as an internal control and it was ubiquitously expressed in all tested organs. The expression of all studied genes showed in at least one tissue. Moreover, most of the selected genes (19 among 25, 76%) were differentially expressed in the different analyzed tissue. For instance, *VfCOMT* and *VfDELLA* were not found in flowers, whereas *VfWRKY* and *VfLEA* were slightly expressed. In the other hand, *VfNod19* mRNA was not found in seeds and stems tissue, however a preferential accumulation of this transcript was revealed in root tissue. *VfLEA6*, *VfWRKY*, *VfPS/TP*, *VfGST*, *VfLEA*, *VfDELLA*, *VfLEA3*, *VfHMT* and *VfPOX* are highly expressed in leaves and lower in other tissues.

Expression patterns of the selected genes under osmotic and salt stress

In order to determine early and genes differentially and strongly induced by osmotic and salt stress treatments,


A
□Bachar ■Giza 3
RWC (%)
Control 3h 6h 12h 24h 48h
B
□Bachar ■Giza 3
RWC (%)
Control 3h 6h 12h 24h 48h
C
□Bachar ■Giza 3
Proline content (µg/mg DW)
Control 3h 6h 12h 24h 48h
D
□Bachar ■Giza 3
Proline content (µg/mg DW)
Control 3h 6h 12h 24h 48h
E
□Bachar ■Giza 3
Soluble sugar content (µg/mg DW)
Control 3h 6h 12h 24h 48h
F
□Bachar ■Giza 3
Soluble sugar content (µg/mg DW)
Control 3h 6h 12h 24h 48h
G
□Bachar ■Giza 3
H2O2 content (µM/g FW)
Control 3h 6h 12h 24h 48h
H
□Bachar ■Giza 3
H2O2 content (µM/g FW)
Control 3h 6h 12h 24h 48h
I
□Bachar ■Giza 3
MDA content (nmol/g FW)
Control 3h 6h 12h 24h 48h
J
□Bachar ■Giza 3
MDA content (nmol/g FW)
Control 3h 6h 12h 24h 48h

◂**Fig.1** Effects of PEG and NaCl treatments on relative water content (**a**, **b**, respectively), proline (µg/mg DW) (**c**, **d**, respectively), soluble sugars (µg/mg DW) (**e** and **f** respectively), H_2O_2 (µM/g FW) (**g**, **h**, respectively) and MDA (nmol/g FW) content (**i**, **j**, respectively) in leaves of Bachar and Giza 3. The values are means (±SD) of four replicates. Letters above the bars represent significant differences at $P < 0.05$ (Tukey's pairwise comparison)

real-time PCR was carried out to analyze gene expression patterns of selected genes by using fold-change values transformed to Log2 of the fold difference in expression in leaves and roots stressed samples as compared to the corresponding control at 0 h time point. The hierarchical clustering of the genes is represented in heat maps which were performed to shown the expression patterns of selected genes in response to osmotic and salt stress. The white rectangles in the heatmap represent the gene showing maximum expression at a particular time point. Differential regulation of the majority of selected genes was shown in Fig. 3 under osmotic and salt stress in leaves tissue during the time-course. Obtained data indicate significant differences between gene expression patterns at every time point. Overall, significant differences in gene expression level were observed after 3 h of osmotic and salt stress treatments, suggesting a quick transcriptional regulation in both studied genotypes to the abiotic stress response. Most candidate genes showed high expression in osmotic and salt-stressed plants (Bachar and Giza 3), although some significant differences could also be observed. For the osmotic treatment (Fig. 3a), studied genes could be broadly divided into four clusters. The first cluster contains 8 (32%) members (*VfERF*, *VfPOX*, *VfRD22*, *VfDELLA*, *VfLEA3*, *VfLEA*, *VfNAC* and *VfCIPK*) of selected abiotic response genes, which were widely upregulated after 3 h by osmotic treatment, and the maximum induction being recorded at 6 h of PEG treatment. Cluster 2 has 10 (40%) members (*VfMATE*, *VfHMT*, *VfDHN*, *VfPS/TP*, *VfUDP*, *VfGST*, *VfLEA6*, *VfMEM*, *VfERF-B3* and *VfERD*). Indeed, *VfMATE*, *VfHMT*, *VfDHN*, *VfPS/TP* and *VfERD* showed upregulated response at 3 or 6 h, but downregulated at 12, 24 and 48 h after osmotic treatment. However, *VfUDP*, *VfGST*, *VfLEA6*, *VfMEM* and *VfERF-B3* showed a weakly upregulated expression at 3 or 6 h of stress, while at the other time spans, their expression remained unchanged. Cluster 3 contains 3 (12%) genes (*VfTK*, *VfWRKY* and *VfNod19*), which were mainly upregulated with the increased level of osmotic treatment after 3 h, and the *VfTK* in particular was highly induced by PEG treatment in Bachar and Giza 3 at 6 and 24 h, respectively. However, *VfWRKY* exhibited high expression level at 3 and 6 h respectively. In the other hand *VfNod19* showed high level of expression at 12 and 24 h in Bachar and Giza 3 respectively. Cluster 4 mainly consists of 4 (16%) genes (*VfMYB-related*, *VfAP2LP*, *VfCOMT* and *VfMYB*), which were widely downregulated by osmotic treatment (Fig. 3a).

For the salt stress treatment, the heatmap displayed five clusters of genes (Fig. 3b). Most (9, 36%) members (*VfMATE*, *VfDHN*, *VfPS/TP*, *VfGST*, *VfLEA3*, *VfCIPK*, *VfNAC*, *VfWRKY*, and *VfPOX*) of cluster 1 were mainly upregulated after the salt treatment and exhibited the highest expression at 12 and 48 h in Bachar and at 6 h in Giza 3. The 7 (28%) members (*VfLEA*, *VfLEA6*, *VfMEM*, *VfHMT*, *VfUDP*, *VfERF-B3* and *VfAP2LP*) of cluster 2 were weakly upregulated or showed a relatively stable expression pattern under salt stress. Cluster 3 contained the smallest (2, 8%) members (*VfTK* and *VfNod19*) which were downregulated in continuation of salt stress for 48 h. The 4 (16%) members (*VfERF*, *VfRD22*, *VfDELLA,* and *VfERD*) of cluster 4 were mainly upregulated at 3 and 6 h of salt treatment but were downregulated after 12 h in Bachar. A similar expression of the members of cluster 4 in Giza 3 was observed, but the differences that they were upregulated at 6 and 12 h of salt stress and then downregulated at 24 h and 48 h of salt stress. Cluster 5 has 3 (12%) members (*VfMYB-related*, *VfCOMT,* and *VfMYB*) of the 25 studied genes, which were widely downregulated at 3, 6, and 24 h in leaves tissue of Bachar and at 3, 12, 24 and 48 h in Giza 3.

The expression of the selected genes in root tissues of Bachar and Giza 3 showed also different trends related to the duration of osmotic and salt stress (Fig. 4). Based on a hierarchical clustering analysis, the 25 studied genes were mainly clustered into three clusters when roots of both studied genotypes were subjected to osmotic stress, as shown in Fig. 4a. The first cluster represented 9 (36%) genes (*VfTK*, *VfRD22*, *VfPS/TP*, *VfLEA3*, *VfMEM*, *VfDHN*, *VfAP2LP*, *VfUDP* and *VfNod19*) which were found to be upregulated under osmotic challenge. The maximum accumulation of the first 6 members of the cluster was recorded after osmotic stress for 3, 6 and 12 h in both Bachar and Giza 3, while *VfAP2LP*, *VfUDP* and *VfNod19* showed a striking induction at 24 and 48 h. The induction of *VfTK*, *VfRD22*, *VfPS/TP*, *VfLEA3*, *VfMEM* and *VfDHN* was noted especially in Bachar, while the induction in *VfAP2LP*, *VfUDP* and *VfNod19* was recorded especially in Giza 3. The second cluster gathered together 14 (56%) genes (*VfERF*, *VfWRKY*, *VfNAC*, *VfERD*, *VfGST*, *VfMATE*, *VfPOX*, *VfMYB*, *VfHMT*, *VfCOMT*, *VfLEA*, *VfCIPK*, *VfERF-B3* and *VfLEA6*) with a striking induction under osmotic stress at 6 and 12 h; they were found to be downregulated at 24 and 48 h, though there are some exceptions in the expressions of *VfLEA*, *VfCIPK*, *VfERF-B3* and *VfLEA6* which were obviously weakly upregulated or unchanged under osmotic stress. The third cluster was composed of 2 (8%) genes (*VfDELLA* and *VfMYB-related*) which were widely downregulated after PEG treatment.

As shown in Fig. 4b, the expression of all the selected genes in root tissue could be induced or repressed under NaCl stress treatment. The heatmap disclosed the genes distributed in five clusters. Cluster 1 and 2 (11 genes) showed

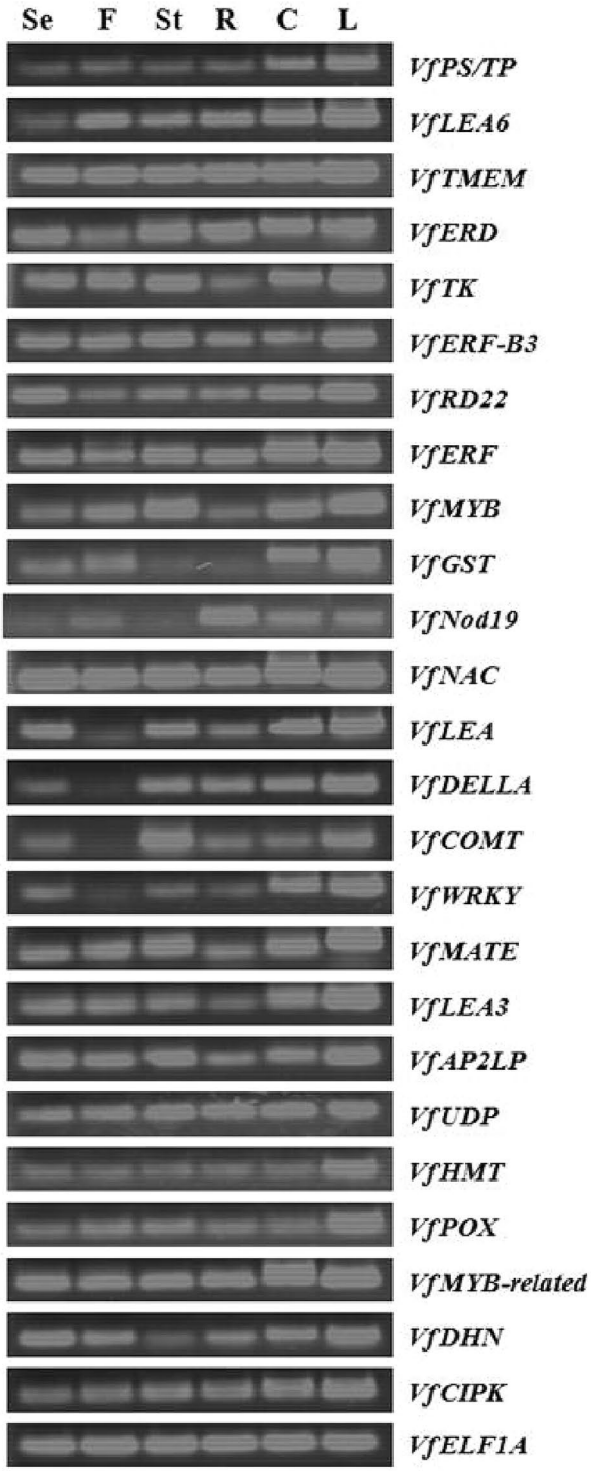


Fig. 2 Tissue-specific expression using semiquantitative RT-PCR analysis of selected genes. RNA was isolated from vegetative tissues, such as leaves (L), cotyledons (C), roots (R), stems (St), as well as reproductive tissues, like flowers (F), and seeds (Se). Transcripts of faba bean elongation factor 1-alpha (*VfELF1A*) were used as a control

most of the upregulated genes. Under salt conditions, all the 5 (20%) genes (*VfLEA3*, *VfTK*, *VfNAC*, *VfMEM* and *VfPS/TP*) of cluster 1 were found to be significantly upregulated at 3, 6 or 12 h in the root tissues of Bachar and at 3 or 48 h in Giza 3. The 6 members (*VfDHN*, *VfPOX*, *VfERF*, *VfMATE*, *VfERD* and *VfGST*) of cluster 2 were mainly upregulated after the NaCl treatment and exhibited the highest expression at 6 and 12 h in the root tissues of Bachar and at 3 and 48 h in Giza 3. However, we found that in Bachar members of cluster 2 were not significantly regulated under salt stress at 3, 24 and 48 h and in Giza 3 at 6, 12 and 24 h, as their expression patterns when compared to the control ones remained unaffected or downregulated in roots tissue. Seven (28%) members (*VfRD22*, *VfLEA*, *VfERF-B3*, *VfUDP*, *VfLEA6*, *VfAP2LP,* and *VfCIPK*) of cluster 3 showed no change or downregulated expression at each time point of the time course in root tissues of Bachar and Giza 3 with respect to the control conditions. The 5 (20%) members (*VfMYB*, *VfWRKY*, *VfHMT*, *VfNod19,* and *VfCOMT*) of cluster 4 were mainly upregulated after 3 h under NaCl treatment but were downregulated especially at 48 h in Bachar and Giza 3. The 2 (8%) members (*VfDELLA* and *VfMYB*-related) of cluster 5 had a similar expression with cluster 4, but the difference was the both *VfDELLA* and *VfMYB*-related were found to be dramatically downregulated at most time points.

Effects of exogenous ABA (abscisic acid) application on the expression patterns of 17 selected genes

Various phytohormones control multiple biological processes and act as regulators of plant response to abiotic stresses. ABA is a central regulator of environmental constraints responses in plants. To assess the differential expression of *VfRD22*, *VfLEA*, *VfLEA6*, *VfAP2LP*, *VfCIPK*, *VfMYB*, *VfWRKY*, *VfMYB-related*, *VfPOX*, *VfDHN*, *VfERF*, *VfUDP*, *VfMATE*, *VfERD*, *VfLEA3*, *VfNAC*, and *VfGST* under treatment with exogenous ABA, their expression profiles were investigated in the leaves (Fig. 5a) and roots (Fig. 5b) tissue of Bachar and Giza 3 using qRT-PCR analyses during 0 (control), 1, 3, 6 and 12 h durations of ABA treatment. Based on hierarchical clustering analysis, the 17 selected genes were mainly clustered into 4 clusters, as shown in Fig. 5a. All the studied genes were responsive to ABA treatment in the two studied genotypes. Interestingly, most of the studied genes revealed a similar expression pattern in Bachar and Giza 3, although some significant differences could also be observed. Cluster 1 contains 4 (23.5%) members (*VfCIPK*, *VfLEA*, *VfERF,* and *VfPOX*) which were widely upregulated after 1 h by ABA treatment, and up to the highest expression level at 12 h (Fig. 5a). Expression of the 4 (23.5%) members (*VfDHN*, *VfMATE*, *VfLEA3* and *VfWRKY*) of cluster 2 was upregulated after 1 h by ABA treatment compared with control but downregulated or nearly

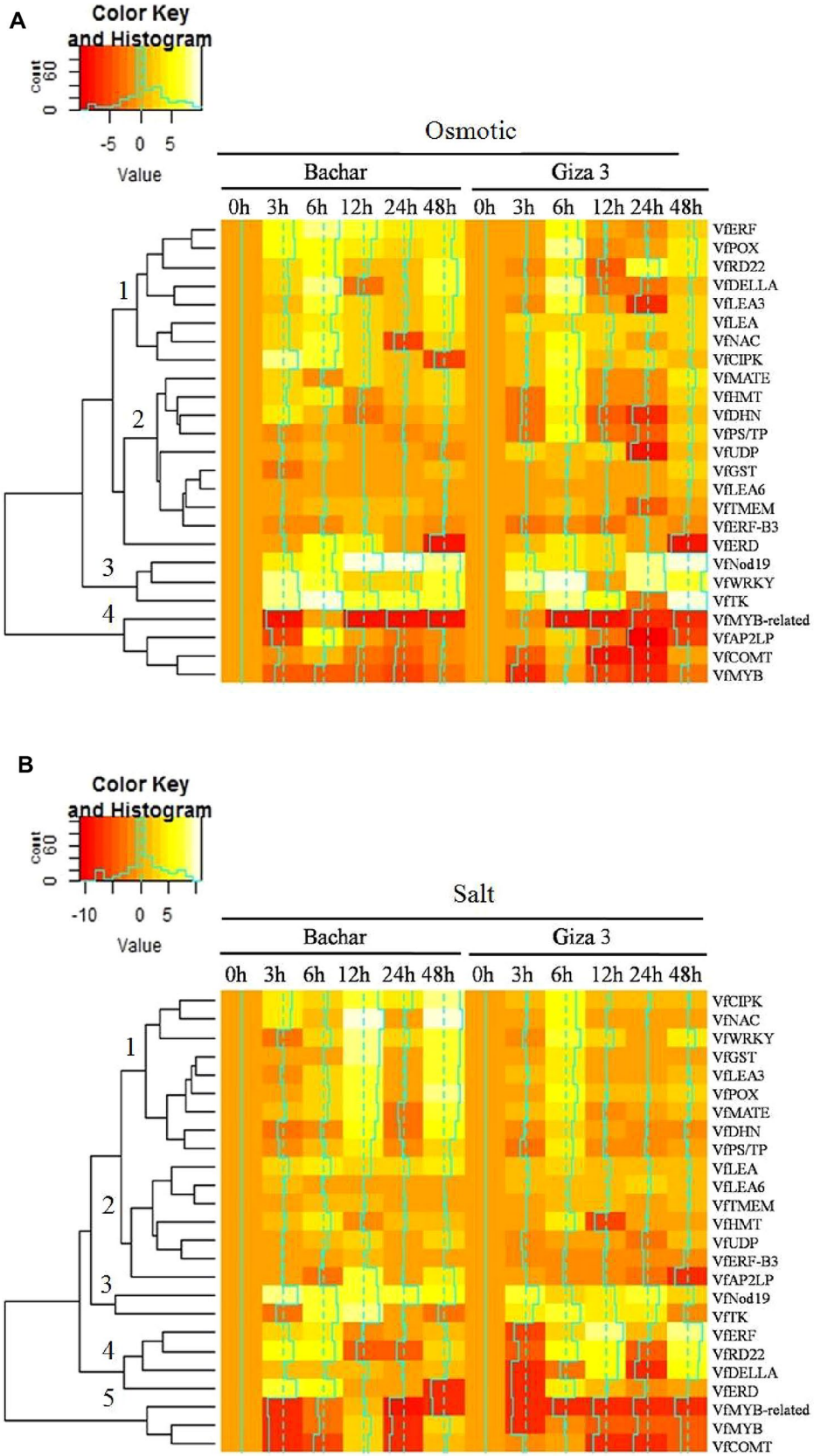


Fig. 3 Heat map representation of the differential expression of the candidate genes in response to PEG-6000 (**a**) and NaCl (**b**) in the leaves of Giza 3 and Bachar at 0 (control), 3, 6, 12, 24 and 48 h after stress induction. White and red indicate higher and lower expression values, respectively. The intensity of the colors is proportional to the absolute value of log2 of the fold difference in gene expression

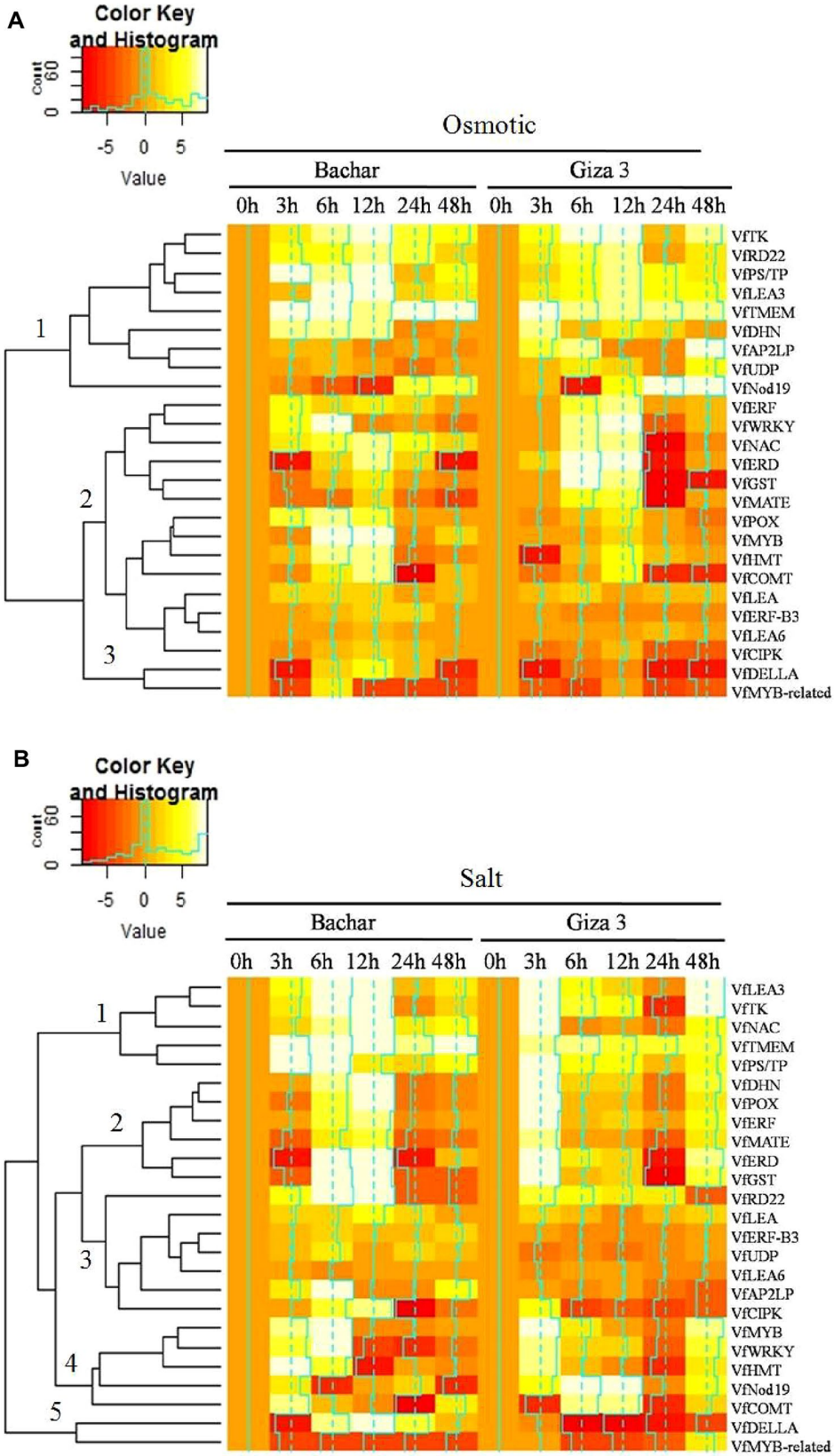


Fig. 4 Heat map representation of the differential expression of the candidate genes in response to PEG-6000 (**a**) and NaCl (**b**) in the roots of Giza 3 and Bachar at 0 (control), 3, 6, 12, 24 and 48 h after stress induction. White and red indicate higher and lower expression values, respectively. Intensity of the colors is proportional to the absolute value of log2 of the fold difference in gene expression

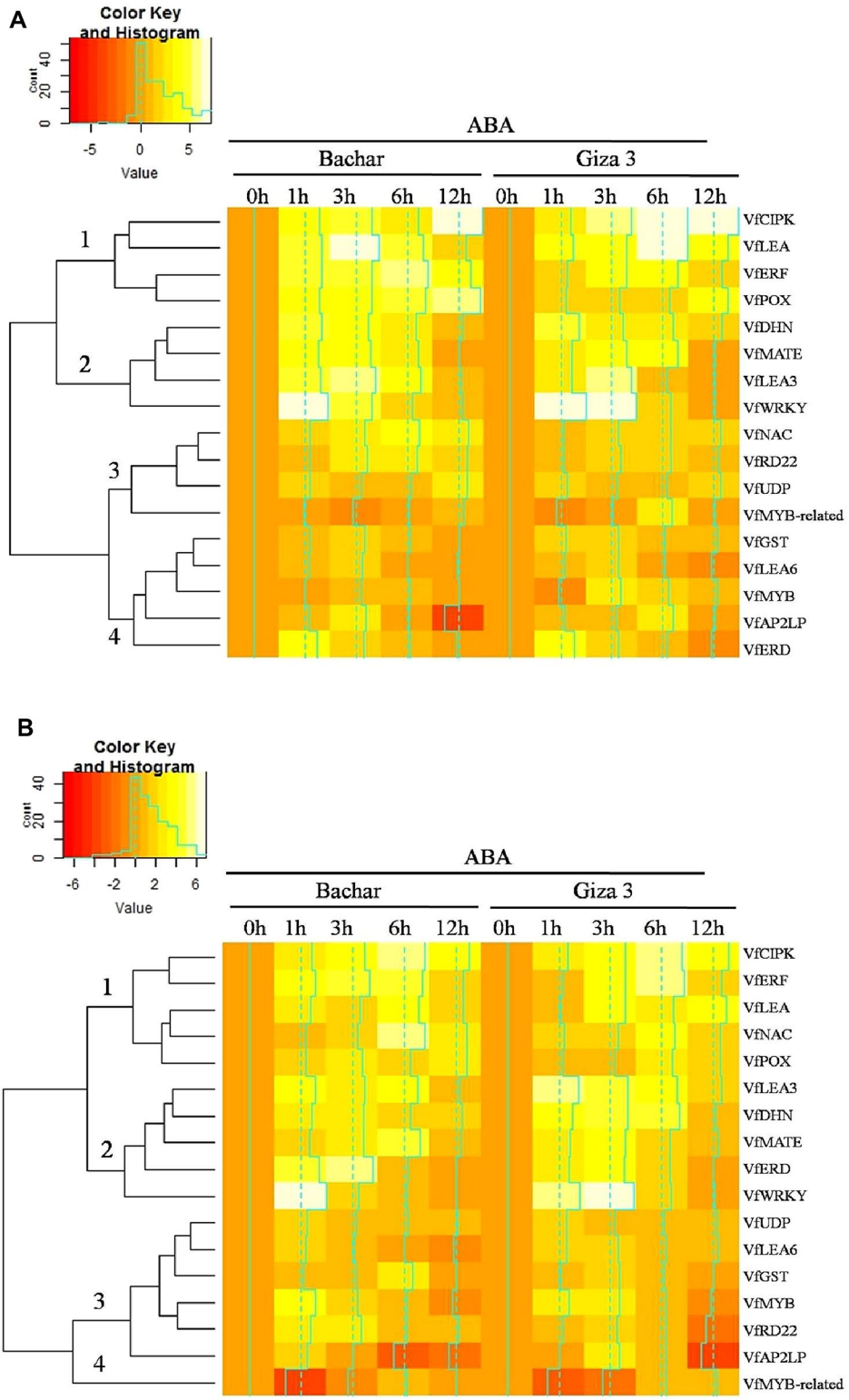


Fig. 5 Heat map representation of the differential expression of 17 selected candidate genes in response to exogenous ABA treatment (100 µM) in leaves (**a**) and roots (**b**) of Giza 3 and Bachar at 0 (control), 1, 3, 6, and 12 h. White and red indicate higher and lower expression values, respectively. Intensity of the colors is proportional to the absolute value of log2 of the fold difference in gene expression

unchanged at 12 h. Similar to cluster 1, cluster 3 consists of 4 (23.5%) genes (*VFNAC*, *VfRD22*, *VfUDP* and *VfMYB*-related) showing upregulated expression in response to the same treatment across all time points, although expressed at low levels. Five members (29.5%) of cluster 4 showed a weakly upregulated at 1, 3 and 6 h, though the expression level was more pronounced at 3 h but downregulated or nearly unchanged at 12 h after ABA treatment (Fig. 5a).

For the ABA treatment of roots, these genes could be divided also into 4 clusters (Fig. 5b). With ABA treatment, the 5 (29.5%) genes (*VfCIPK*, *VfERF*, *VfLEA*, *VfNAC* and *VfPOX*) of cluster 1 showed significantly enhanced transcript accumulations in roots of Bachar and Giza 3, though the expression level was more pronounced at 6 h, where the expression was constitutive (Fig. 5b). The inducibility by ABA was clear from the gene expression pattern of cluster 2 in Bachar and Giza 3, the 5 (29.5%) genes (*VfLEA3*, *VfDHN*, *VfMATE*, *VfERD,* and *VfWRKY*) were widely upregulated after 1 h, then downregulated to reached control levels at 12 h. The highest expression level was shown in *VfWRKY*, which in particular was highly induced at 1 h after ABA treatment in Bachar and at 3 h in Giza 3. Cluster 3, representing a total of 6 (35%) genes (*VfUDP*, *VfLEA6*, *VfGST*, *VfMYB*, *VfRD22* and *VfAP2LP*), were slightly upregulated at 1 and 3 h by ABA treatment in both genotypes but downregulated or nearly unchanged in Bachar at 6 and 12 h. However, expression was nearly unchanged at 6 h but downregulated at 12 h in Giza 3. Moreover, *VfMYB*-related which contains cluster 4 was downregulated by ABA treatment at an early stage (1 and 3 h after treatment) but was weakly upregulated at 6 and 12 h.

Discussion

In this study, physiological and biochemical stress indicators in response to environmental challenges showed that Bachar and Giza 3 displayed a variability of responses to osmotic and salt stress (Fig. 1).

Data revealed that osmotic and salt stress induced by PEG and NaCl, respectively, significantly affected RWC in Bachar and Giza 3. Under osmotic stress at 0, 3, 6 and 12 h, the RWC was recorded slightly highest in Bachar compared to Giza 3. In the other hand, similar results were obtained under salt application, although some significant differences could be observed. Indeed, at 12, 24 and 48 h, the RWC was higher in Bachar, than in Giza 3. Earlier significant decline and greater extent decrease in RWC were found in Giza 3 than in Bachar, which suggested a better osmotic and salt adaptation in Bachar. The results obtained here for *Vicia faba* were almost similar to previous studies (Siddiqui et al. 2015; Abid et al. 2017). Ghuge et al. (2010) also reported that osmotic stress induced by NaCl and PEG significantly reduced the relative water content of the potato plant. Results of this work are in agreement with previous studies in *Vicia faba* (Alghamdi et al. 2015) and other plant species like rice (Swapna and Shylaraj 2017) reporting that osmotic tolerant genotypes showed higher RWC rather than osmotic sensitive genotypes.

Proline is the most widely accumulated osmolyte under abiotic stress and which was correlated with tolerance to environmental constraints. Moreover, its acts as an antioxidant, and stress-related signaling molecule (Hayat et al. 2012). According to these authors, rapid and high amounts of free proline accumulation are a typical plant response to abiotic stress. Data showed that amount of free proline in leaves of Bachar and Giza 3 increased significantly under osmotic and salt stress. Under osmotic stress, proline significantly and particularly accumulated in Bachar at 48 h in comparison with Giza 3. Moreover, under salt stress Bachar plants increased proline content more than Giza 3. This increase was very significant at all time points. Augmentation of proline content in leaf tissues of faba bean due to abiotic stress has been noted in other researches (Siddiqui et al. 2015; Migdadi et al. 2016; Abid et al. 2017). Similar results were reported in several crops such as wheat (Mwadzingeni et al. 2016) and chickpea (Awasthi et al. 2017) which accumulate high level of proline in drought tolerant genotypes than sensitive. Data of the present investigation suggested that prominent accumulation of proline in Bachar constitutes a protective mechanism to decrease oxidative damage reducing osmotic stress in this genotype. According to Hare and Cress (1997), the level of proline accumulated in stressed-plants relatively correlated with the amount of carbohydrates. Indeed, the amount of soluble sugars was positively associated with abiotic stress tolerance in various plant species like faba bean (Siddiqui et al. 2015; Abid et al. 2017), rice (Redillas et al. 2012) and sugar beet (Wu et al. 2014). Current studies validated these findings with enhanced soluble sugars levels in osmotic/salt stressed Bachar and Giza 3 plants in comparison to control. Interestingly, these enhancements were more pronounced in the cultivar Bachar than in the Giza 3 throughout all time points. The amount of soluble sugar accumulated in Bachar and Giza 3 stressed-plants suggesting a potential role in osmotic adjustment, with a better performance of Bachar, particularly in maintaining a better water balance under osmotic stress (Farooq et al. 2009). Indeed, significant variations in free proline and soluble sugars appeared at earlier osmotic and salt stages in Bachar than in Giza 3.

Environmental challenges, contribute to formation of ROS (reactive oxygen species) in the cells. Among the various ROS, H_2O_2 is one of the most abundant in aerobic biological systems, being highly reactive and toxic. H_2O_2 causes perturbation of basic metabolic pathways, damages the membrane lipids, induces protein denaturation and may also inactivate enzymes, induces DNA mutations and at high level, it could lead to cell death (Sharma et al. 2012). As for indicators of oxidative stress, MDA and H_2O_2 contents were determined in leaves of Bachar and Giza 3. In this study, the H_2O_2 contents significantly increased with osmotic and salt stress progressed in the Bachar and Giza 3 cultivars but in contrast with proline and soluble sugars parameters, H_2O_2 has negative effects on osmotic tolerance. Differences were

found in H_2O_2 contents between Bachar and Giza 3 seedlings when exposed to osmotic and salt pressure. At all time points Giza 3 exhibited the highest H_2O_2 content under PEG and NaCl treatments and compared to Bachar, it showed higher increased rate in contents of H_2O_2. Results of this study indicated significant differences among Bachar and Giza 3 for H_2O_2 accumulation and scavenging. These data were similar to the one obtained by Abid et al. (2017) in *Vicia faba*.

Among the lipid peroxidation products, malondialdehyde (MDA) is the most indicator of oxidative damage and used as potential marker to screen the tolerant and susceptible genotypes (Xu et al. 2008). In olive tree and turfgrass, low MDA content was correlated to abiotic stress tolerance (Bacelar et al. 2007; Xu et al. 2013). Leaf MDA content increased significantly in Bachar and Giza 3 osmotic and salt-stressed plant showing that the salinization and dehydration of faba bean is associated with lipid peroxidation mechanisms. Giza 3 had the highest MDA content at 3 and 6 h after PEG treatment, while the highest MDA content under salt stress appeared at 6 h after NaCl treatment. Results of this study suggested less severe lipid membrane damage in Bachar than Giza 3. Thus, lower level in leaves of Bachar suggests better protection of this cultivar against the peroxidation of the membrane under abiotic stress. The results are in good agreement with previous work, in which higher MDA have been reported in sensitive faba bean genotypes compared to the tolerant ones (Siddiqui et al. 2015; Abid et al. 2017). Similar results were reported by Chugh et al. (2013) in maize. Compared with Bachar, Giza 3 possessed a higher increased rate in contents of MDA and H_2O_2 and earlier significant changes were found in Giza 3, which suggested that Bachar possessed better osmotic tolerance and stronger osmotic adaptation.

The results indicated that Bachar and Giza 3 respond differentially to osmotic and salt exposure induced by PEG and NaCl. Compared with Giza 3, Bachar had the lowest MDA and H_2O_2 content and higher RWC. In the other hand, proline and soluble sugars content was the highest in this cultivar which suggest that Bachar was less affected than Giza 3 under these stress conditions. These data suggest that Bachar is more resistant to NaCl and PEG-induced osmotic stress than Giza 3. These observations are in agreement with some reports demonstrating that osmotic response of faba bean is greatly affected by genetic factors (Siddiqui et al. 2015; Migdadi et al. 2016; Abid et al. 2017).

In a context of unprecedented climate change, the development of drought and salt-tolerant cultivars is one of the most significant challenges of traditional *Vicia faba* breeding programs in the last decade and critical to sustain this grain legume production, particularly in the Mediterranean ecosystem. The traditional breeding approach limited the development of faba bean cultivars for abiotic stress tolerance. Indeed, plant response to abiotic stress is a complex trait involving several genes related with various cellular signaling pathways. Therefore, it is imperative to first identify genes involved in osmotic stress tolerance for understand the mechanism of abiotic stress tolerance. This may be lead to create new crop varieties with increased tolerance to environmental constraints. In recent years, several transcriptomic approaches including RNA deep sequencing and microarray (Hossain et al. 2016; Lu et al. 2017) have been employed to elucidate the differential expression of RNA transcripts involved in osmotic response which leads to increased knowledge about osmotic signaling pathways. Interestingly, for plants whose genomes have not been completely or partially sequenced, computational approaches such as in silico analysis provide a good way for discovering genes involved in plant stress tolerance (Ram and Sharma 2013).

Based on microarray data in salt-stressed *Medicago truncatula* plants (Li et al. 2009), an in silico analysis was performed in order to identify gene networks involved in faba bean abiotic stress tolerance. A total of 123 and 104 faba bean genes showing homology with upregulated and downregulated salt stress response genes in *Medicago truncatula*, respectively were determined. Among the identified genes, 25 (Table 1) were selected. These genes showed different functional categories including ABA-responsive. Their expression patterns were analyzed by real time PCR in leaves and roots tissue of Bachar and Giza 3. Some of the key genes are classified as transcription factors like *WRKY*, *NAC*, *ERF*, *AP2LP* and *MYB*. Others consist of functional proteins related to abiotic stress tolerance including *LEA* and *CIPK* or ROS detoxify enzymes like *GST* and *POX*. Most of the selected genes differentially expressed in response to abiotic stress, suggesting a potential role in the modulation of drought and salt stress tolerance. Moreover, transgenic lines over-expressing some of those genes, such as *Tamarix hispida* expressing *GST* (Yang et al. 2014), wheat plants over-expressing *DHN-5* (Brini et al. 2007) or *ERF* (Rong et al. 2014), transgenic lines of *Arabidopsis* expressing *TaWRKY1* and *TaWRKY33* (Mao et al. 2011) or *TaMYB2A* (He et al. 2016) and transgenic rice plants expressing *NAC* transcription factor (Hong et al. 2016) showed increased tolerance to drought and salt stress. Indeed, expression patterns of these genes have been carried out in several plants under drought and salt constraint but have not been previously studied in *Vicia faba*. PCR data showed that most selected genes were expressed in various organs (leaf, root, flower, stem, cotyledon, and seed) of faba bean, but showed different spatial transcript accumulation pattern. This indicates that they may participate in growth and development and each member might play a particular physiological function.

Most selected genes showed significant differences in the relative mRNA levels between Bachar and Giza 3 under PEG, NaCl and ABA treatments. Indeed, under salt and osmotic stress, gene encoding *VfPOX*, *VfLEA3*, *VfNAC*,

VfCIPK, *VfLEA*, *VfWRKY*, *VfERF*, *VfRD22*, *VfDELLA*, *VfNod19,* and *VfTK* showed constitutive upregulation in the leaves tissue at all examined time points, while *VfMYB*-related, *VfMYB*, *VfCOMT* and *VfAP2LP* showed constitutive downregulation. The remaining genes (*VfERD*, *VfMATE*, *VfGST*, *VfDHN*, *VfPS/TP*, *VfLEA6*, *VfTMEM* and Vf*ERF-B3*) were upregulated or downregulated at only specific time points, indicating a temporal control of gene transcription under osmotic stress. In general, the expression of these genes was upregulated in the leaves of the tolerant cultivar Bachar. In the root tissues of PEG and NaCl-stressed Bachar and Giza 3 plants, expressions for 7 genes (*VfPOX*, *VfLEA3*, *VfNAC*, *VfWRKY*, *VfERF*, *VfRD22*, and *VfTK*) out of 11 induced genes in leaves showed upregulated in a constitutive manner. Moreover, *VfDHN*, *VfPS/TP*, *VfHMT* and *VfMYB* were also found to show upregulation under all the examined conditions, whereas *VfMYB*-related, *VfCIPK* and *VfDELLA* showed downregulation. The rest of the genes are either expressed or turned off under specific conditions. Current evidence links changes in ABA levels and the expression of specific ABA-reponsive genes related to environmental stress response and tolerance. ABA application induced the expression of 16 out of 17 (94%) of the studied genes in the leaves and roots tissue of Bachar and Giza 3 compared to control, whereas the expression of the remaining gene (*VfMYB*-related) significantly downexpressed. Results of this study were in accordance with previous reports, which found that most of the studied genes are involved directly or indirectly in abiotic stresses responses in plants and many of them have been reported as key regulators of plant response to osmotic stress. The *WRKY* transcription factors play crucial roles in growth, development and abiotic stresse response in plant. Indeed, some WRKY genes like Arabidopsis *WRKY8* (Hu et al. 2013), Moso bamboo *PeWRKY83* (Wu et al. 2017), cotton *GhWRKY68* (Jia et al. 2015) and soybean *GmWRKY21/54* (Zhou et al. 2008) were upregulated by drought, salt or ABA treatment. Interestingly, gain-of-function of these *WRKY* genes enhanced transgenic lines' tolerance to osmotic stress. Exposed to salinity, 10 *GSTs* and one *POX* genes encoding antioxidant enzymes were upregulated in root tissues of citrus plants and played important protective roles in coping with excessive accumulation of ROS (Xie et al. 2018). Expression profiles of *VfGST* and *VfPOX* suggest their role in stress adaptation and tolerance towards various abiotic stresses.

In plants, the MYB (myeloblastosis) proteins constitute one of the largest transcription factors family which play a number of roles in regulating plant growth, metabolism and various stress responses. Dubos et al. (2010) classified MYB proteins into 4 classes (1R-MYB, R2R3-MYB, 3R-MYB and 4R-MYB) based to the number of conserved MYB DNA-binding domain. Overall, 155 and 197 *MYB* genes were identified in *Oryza sativa* and *Arabidopsis thaliana* and among them; several members are upregulated by various abiotic stresses. Overall, 155 and 197 *MYB* genes were identified in rice and Arabidopsis and among them; several members are upregulated by various abiotic stresses. Indeed, using real time PCR, the expression of 28 *OsMYB* genes among 60 showed significantly increased under drought stress in rice. However, among the 21 *MYB* genes analyzed in *Arabidopsis* 7 *AtMYB* were upregulated and 7 *AtMYB* were downregulated (Katiyar et al. 2012). In watermelon, exogenous ABA application induced expression of 19 *MYB* genes, whereas the 4 rest genes showed repressed expression in comparison with the control (Xu et al. 2017). The *MYB* gain-of-function mutants in rice (Yang et al. 2012) and Arabidopsis (Ding et al. 2008) showed increased resistance to abiotic stress. These results indicated that a large number of *MYB* genes could play a role against abiotic stress in rice and Arabidopsis. *VfMYB* exhibited significantly reduced expression in leaves tissue under PEG and NaCl treatment. However, *VfMYB-related* showed significantly reduced transcript levels under PEG, NaCl and ABA treatment in leaves and roots of Bachar and Giza 3. This could suggest the involvement of *VfMYB-related* in the crosstalk of different signaling and metabolic pathways. This was in line with early results found in watermelon reported by Xu et al. (2017). These authors revealed a remarkably reduction in the expression of *ClaMYB1R7* gene encoding *MYB*-related class under salinity and exogenous application of ABA.

The AP2/ERF family is a large gene family transcription factors which is unique to plant and they play major roles not only in plant growth, development and metabolite biosynthesis but also are considered as central regulators in cold, drought, salt and heat stress responses (Gu et al. 2017). According to the classification adopted by Licausi et al. (2010) based on the difference of the common DNA binding domain copy numbers, the superfamily AP2/ERF includes five subfamilies: AP2 (APETALA2), ERF (ethyleneresponsive-element-binding-factor) which could be classified into six groups (B1–B6), DREB (dehydration responsive element binding), RAV (related to ABI3/VP) and other proteins (Soloist). Generally, AP2/ERF members are stimulated by ABA signals and can be induced in plants by different abiotic stresses (Gu et al. 2017). Rice, tomato and tobacco transgenic lines overexpressing *ERF* members showed enhanced resistance to drought stress and salinity (Guo et al. 2004; Lu et al. 2010; Zhang et al. 2010). Moreover, mutants tomato and wheat overexpressed *SlERF5* (Pan et al. 2012) and *TaERF3* (Rong et al. 2014) exhibited significantly enhanced tolerance to abiotic stress. Obtained data showed that faba bean *AP2/ERF* members differentially expressed suggesting a crucial role in abiotic stress and ABA response. Indeed, *VfERF* and *VfERF-B3* were upregulated, while *VfAP2LP* was significantly downregulated particularly in leaves tissue under osmotic and ABA treatments.

Recently, Dossa et al. (2016) reported similar results in sesame regarding the transcripts accumulation of *AP2/ERF* under water deficit. Indeed, this environmental stress has decreased the transcript abundance of *AP2si115*, *AP2si47*, *AP2si103* and *AP2si11*.

VfNAC transcription factor was also found to be strongly and differentially induced in leaves and roots of Bachar and Giza 3 in response to different treatments. This suggested a potential usefulness of *VfNAC* in improving abiotic stress resistance of faba bean. Various *NAC* transcription factors have been documented as central regulators during plant stress response (Puranik et al. 2012). Moreover, several *NAC* genes such as *OsNAC6* were induced by ABA, cold, salt and drought stresses (Ohnishi et al. 2005). Overexpressing of rice *OsNAC6* gene conferred dehydration and enhanced resistance to salinity (Nakashima et al. 2007). More recently, in transgenic rice overexpression of *ONAC022* also caused an increase in drought and salt stress tolerance (Hong et al. 2016).

In plants, late embryogenesis abundant (LEA) proteins form a large family associated with plant responses and adaption to environmental stress, that includes seven different groups (LEA1–LEA7) based on their sequence similarities, repeated motifs and amino acid composition (Battaglia et al. 2008). In different plant species, genes called dehydrins (*DHNs*), responsive to dehydration (*RD*), early responsive to dehydration (*ERD*), cold-inducible (*KIN*), cold regulated (*COR*) and responsive to ABA (*RAB*) encodes for LEA type proteins have been reported previously (Zhu 2002). It is well known that LEA proteins are potentially expressed under different environmental stresses like salinity, drought, cold and heat as well as ABA treatment. Moreover, overexpression of LEA proteins from various groups showed improved tolerance to abiotic stress. Indeed, rice plants overexpressed *OsLEA4* gene exhibited high tolerance to various stresses such as drought, salinity and heavy metals (Hua et al. 2016).

In the other hand, overexpression of dehydrine gene (*OsDhn1*) in rice improved its tolerance to drought and salt stress by effectively scavenging ROS (Kumar et al. 2014). In this work, *VfLEA*, *VfLEA3*, *VfLEA6*, *VfDHN* and *VfERD* genes were induced under PEG, NaCl, and ABA treatments, but showed completely different expression patterns among Bachar and Giza 3 suggesting their potential role in salt and osmotic stresses response in faba bean. Multiple genes encoding calcium sensors in plants decode Ca^{2+} signals including calcium-dependent protein kinases (CDPKs), calmodulin-dependent protein kinases (CaMKs), calmodulin-like proteins (CML), calmodulin (CaM) and calcineurin B-like [CBL] protein interaction protein kinase (CIPK) have been shown to be involved in the regulation of abiotic stress response in plants (Das and Pandey 2010). According to Kim et al. (2003), ABA, salinity, drought and cold treatment resulted in the higher accumulation of *CIPK3* transcript in Arabidopsis. Moreover, drought, ABA, PEG, cold, salt and heat treatment significantly induced the expression of *ZmCIPK16* in maize (Zhao et al. 2009). Interestingly, rice plants overexpressed *OsCIPK03*, *OsCIPK12* and *OsCIPK15* gene exhibited a higher cold, drought and salt tolerance (Xiang et al. 2007). Overall, transcripts accumulation of *VfCIPK* gene showed increased after treatment with PEG, NaCl and ABA and showed different trends related to the duration of stress exposure in Bachar and Giza 3, indicating their possible function in *Vicia faba* abiotic stress response.

Conclusion

From this study, we can conclude that PEG (21%) and NaCl (200 mM) treatments affected significantly physiological and biochemical parameters, with lesser effects on Bachar compared to Giza 3. Indeed, Bachar showed greater accumulation of proline and soluble sugars and less accumulation of MDA and H_2O_2 under osmotic and salt stress, suggested that this cultivar exhibited higher osmotic and salt tolerance than Giza 3 through the accumulation of osmoprotectants and a better antioxidant machinery performance under osmotic stress. Analysis of expression profiles showed a differential transcriptional regulation of the selected genes in leaves and roots tissue of Bachar and Giza 3. The latter is an aspect of the varietal differences in osmotic stress tolerance, suggesting also that these genes might function collaboratively and play crucial roles to modulate faba bean plant response and tolerance to different environmental stresses. These results may contribute to decipher the transcriptional regulation signaling networks in response to osmotic and salt stress in faba bean.

Author contribution statement GA: performing the experiments, conducted research, and writing of the manuscript. MNS, RNO and YM: planning of the analysis and correcting of the manuscript. SHJ, EG, KS, J-PB, MEA, SE, FB and MJ: helped in the analysis of the results and editing the manuscript. All the authors have read and approved the final manuscript.

References

Abdellatif KF, El Absawy SA, Zakaria AM (2012) Drought stress tolerance of faba bean as studied by morphological traits and seed storage protein pattern. J Plant Stud 2:47–54

Abid G, Hessini K, Aouida M, Aroua I, Baudoin JP, Muhovski Y, Mergeai G, Sassi K, Machraoui M, Souissi F, Jebara M (2017) Agro-physiological and biochemical responses of faba bean (*Vicia faba* L. var. '*minor*') genotypes to water deficit stress. Biotechnol Agron Soc Environ 21:146–159

Alghamdi SS (2007) Genetic behavior of some selected faba bean genotypes. Afr Crop Sci Conf Proc 8:709–714
Alghamdi SS, Al-Shameri AM, Migdadi HM, Ammar MH, El-Harty EH, Khan MA, Farooq M (2015) Physiological and molecular characterization of faba bean (*Vicia faba* L.) genotypes for adaptation to drought stress. J Agro Crop Sci 201:401–409
Al-Suhaibani NA (2009) Influence of early water deficit on seed yield and quality of faba bean under arid environment of Saudi Arabia. Am-Eurasian J Agric Environ Sci 5:649–654
Amede T, Schubert S (2003) Mechanisms of drought resistance in grain legumes. I. Osmotic adjustment. Ethiop J Sci 26:37–46
Awasthi R, Gaur P, Turner NC, Vadez V, Siddique KHM, Nayyar H (2017) Effects of individual and combined heat and drought stress during seed filling on the oxidative metabolism and yield of chickpea (*Cicer arietinum*) genotypes differing in heat and drought tolerance. Crop Pasture Sci 68:823–841
Bacelar EA, Santos DL, Moutinho-Pereira JM, Lopes JI, Gonçalves BC, Ferreira TC, Correia CM (2007) Physiological behaviour, oxidative damage and antioxidative protection of olive trees grown under different irrigation regimes. Plant Soil 292:1–12
Bates LS, Waldern RP, Teare ID (1973) Rapid determination of free proline for water stress studies. Plant Soil 39:205–207
Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol 148:6–24
Brini F, Hanin M, Lumbreras V, Amara I, Khoudi H, Hassairi A, Pages M, Masmoudi K (2007) Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. Plant Cell Rep 26:2017–2026
Büyük I, Aras S (2017) Genome-wide in silico identification, characterization and transcriptional analysis of the family of growth-regulating factors in common bean (*Phaseolus vulgaris* L.) subjected to polyethylene glycol-induced drought stress. Arch Biol Sci 69:5–14
Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116
Chugh V, Kaur N, Grewal MS, Gupta AK (2013) Differential antioxidative response of tolerant and sensitive maize (*Zea mays* L.) genotypes to drought stress at reproductive stage. Indian J Biochem Biophys 50:150–158
Das R, Pandey GK (2010) Expressional analysis and role of calcium regulated kinases in abiotic stress signaling. Curr Genom 11:2–13
De Costa WAJM, Dennett MD, Ratnaweera U, Nyalemegbe K (1997) Effects of different water regimes on field-grown determinate and indeterminate faba bean (*Vicia faba* L.). I. Canopy growth and biomass production. Field Crops Res 49:83–93
Ding Z, Li S, An X, Liu X, Qin H, Wang D (2008) Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. Cell Res 18:1047–1060
Dossa K, Wei X, Li D, Fonceka D, Zhang Y, Wang L, Yu J, Boshou L, Diouf D, Cissé N, Zhang X (2016) Insight into the AP2/ERF transcription factor superfamily in sesame and expression profiling of DREB subfamily under drought stress. BMC Plant Biol 16:171. https://doi.org/10.1186/s12870-016-0859-4
Downey LA, Miller JW (1971) Rapid measurements of relative turgidity in maize (*Zea mays* L.). New Phytol 70:555–560
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 38:350–356
Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010) MYB transcription factors in Arabidopsis. Trends Plant Sci 15:573–581
FAOSTAT (2018). http://www.fao.org/faostat/en/#data/QC. Accessed 24 June 2019
Farooq M, Wahid A, Lee DJ (2009) Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties. Acta Physiol Plant 31:937–945
Ghassemi-Golezani K, Ghanehpoor S, Dabbagh Mohammadi-Nasab A (2009) Effects of water limitation on growth and grain filling of faba bean cultivars. J Food Agric Environ 7:442–447
Ghuge SA, Rai A, Suprasanna P (2010) Comparative effects of NaCl, PEG, Mannitol iso-osmotic stress on solute accumulation and antioxidant enzyme system in potato (*Solanum tuberosum* L.). Plant Stress 4:50–55
Gu C, Guo ZH, Hao PP, Wang GM, Jin ZM, Zhang SL (2017) Multiple regulatory roles of AP2/ERF transcription factor in angiosperm. Bot Stud. https://doi.org/10.1186/s40529-016-0159-1
Guo ZJ, Chen XJ, Wu XL, Ling JQ, Xu P (2004) Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. Plant Mol Biol 55:607–618
Hare PD, Cress WA (1997) Metabolic implications of stress- induced proline accumulation in plants. Plant Growth Regul 21:79–102
Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments. Plant Signal Behav 7:1456–1466
He GH, Xu JY, Wang YX, Liu JM, Li PS, Chen M, Ma YZ, Xu ZS (2016) Drought-responsive WRKY transcription factor genes TaWRKY1 and TaWRKY33 from wheat confer drought and/or heat resistance in Arabidopsis. BMC Plant Biol 16:116–131
Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198
Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Station 347:1–32
Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604–611
Hong Y, Zhang H, Huang L, Li D, Song F (2016) Overexpression of a stress-responsive NAC transcription factor gene ONAC022 improves drought and salt tolerance in rice. Front Plant Sci. https://doi.org/10.3389/fpls.2016.00004
Hossain MR, Bassel GW, Pritchard J, Sharma GP, Ford-Lloyd BV (2016) Trait specific expression profiling of salt stress responsive genes in diverse rice genotypes as determined by modified significance analysis of microarrays. Front Plant Sci. https://doi.org/10.3389/fpls.2016.00567
Hu Y, Chen L, Wang H, Zhang L, Wang F, Yu D (2013) Arabidopsis transcription factor WRKY8 functions antagonistically with its interacting partner VQ9 to modulate salinity stress tolerance. Plant J 74:730–745
Hua T, Zhua S, Tana L, Qib W, Hea S, Wanga G (2016) Overexpression of OsLEA4 enhances drought, high salt and heavy metal stress tolerance in transgenic rice (*Oryza sativa* L.). Environ Exp Bot 123:68–77
Jia H, Wang C, Wang F, Liu S, Li G, Guo X (2015) GhWRKY68 reduces resistance to salt and drought in transgenic *Nicotiana benthamiana*. PLoS ONE 10:e0120646
Katiyar A, Smita S, Lenka SK, Rajwanshi R, Chinnusamy V, Bansal KC (2012) Genome-wide classification and expression analysis of MYB transcription factor families in rice and Arabidopsis. BMC Genom 13:544–563
Khan HR, Paull JG, Siddique KHM, Stoddard FL (2010) Faba bean breeding for drought-affected environments: a physiological and agronomic perspective. Field Crops Res 115:279–286
Khan F, Chai HH, Ajmera I, Hodgman C, Mayes S, Lu C (2017) A transcriptomic comparison of two bambara groundnut landraces under dehydration stress. Genes. https://doi.org/10.3390/genes8040121

Kim KN, Cheong YH, Grant JJ, Pandey GK, Luan S (2003) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in Arabidopsis. Plant Cell 15:411–423

Krannich CT, Maletzki L, Kurowsky C, Horn R (2015) Network candidate genes in breeding for drought tolerant crops. Int J Mol Sci 16:16378–16400

Kumar M, Lee SC, Kim JY, Kim SJ, Aye SS, Kim SR (2014) Overexpression of dehydrin gene, OsDhn1, improves drought and salt stress tolerance through scavenging of reactive oxygen species in rice (*Oryza sativa* L.). J Plant Biol 57:383–393

Li D, Su Z, Dong J, Wang T (2009) An expression database for roots of the model legume *Medicago truncatula* under salt stress. BMC Genom. https://doi.org/10.1186/1471-2164-10-517

Li S, Fan C, Li Y, Zhang J, Sun J, Chen Y, Tian C, Su X, Lu M, Liang C, Hu Z (2016) Effects of drought and salt-stresses on gene expression in *Caragana korshinskii* seedlings revealed by RNA-seq. BMC Genom 17:200. https://doi.org/10.1186/s12864-016-2562-0

Licausi F, Giorgi FM, Zenoni S, Osti F, Pezzotti M, Perata P (2010) Genomic and transcriptomic analysis of the AP2/ERF superfamily in *Vitis vinifera*. BMC Genom. https://doi.org/10.1186/1471-2164-11-719

Lu C, Li Y, Chen A, Li L, Zuo J, Tian H, Luo Y, Zhu B (2010) LeERF1 improves tolerance to drought stress in tomato (*Lycopersicon esculentum*) and activates downstream stress-responsive genes. Afr J Biotech 9:6294–6300

Lu X, Zhou X, Cao Y, Zhou M, McNeil D, Liang S, Yang C (2017) RNA-seq analysis of cold and drought responsive transcriptomes of *Zea mays* ssp. *mexicana* L. Front Plant Sci. https://doi.org/10.3389/fpls.2017.00136

Maazaoui H, Drevon JJ, Sifi B (2016) Improvement of Faba bean (*Vicia faba*e L. var. *minor*) phosphorus uptake and nitrogen fixation in a Tunisian multi local field test. J New Sci Agric Biotechnol 31:1806–1811

Mao X, Jia D, Li A, Zhang H, Tian S, Zhang X, Jia J, Jing R (2011) Transgenic expression of TaMYB2A confers enhanced tolerance to multiple abiotic stresses in Arabidopsis. Funct Integr Genom 11:445–465

Mhamdi R, Jebara M, Aouani ME, Ghrir R, Mars M (1999) Genotypic diversity and symbiotic effectiveness of rhizobia isolated from root nodules of *Phaseolus vulgaris* L. grown in Tunisian soils. Biol Fertil Soil 28:313–320

Migdadi HM, El-Harty EH, Salamh A, Kha MA (2016) Yield and proline content of faba bean genotypes under water stress treatments. J Animal Plant Sci 26:1772–1779

Mukherjee SP, Choudhuri MA (1983) Implications of water stress-induced changes in the leaves of endogenous ascorbic acid and hydrogen peroxide in vigna seedlings. Physiol Plant 58:166–170

Muthuramalingam P, Krishnan SR, Pothiraj R, Ramesh M (2017) Global transcriptome analysis of combined abiotic stress signaling genes unravels key players in *Oryza sativa* L.: an in silico approach. Front Plant Sci. https://doi.org/10.3389/fpls.2017.00759

Mwadzingeni L, Shimelis H, Tesfay S, Tsilo TJ (2016) Screening of bread wheat genotypes for drought tolerance using phenotypic and proline analyses. Front Plant Sci. https://doi.org/10.3389/fpls.2016.01276

Nadeem M, Li J, Yahya M, Sher A, Ma C, Wang X, Qiu L (2019) Research progress and perspective on drought stress in legumes: a review. Int J Mol Sci 20:2542. https://doi.org/10.3390/ijms20102541

Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano HY, Tsutsumi N (2005) OsNAC6, a member of the NAC gene family, is induced by various stresses in rice. Genes Genet Syst 80:135–139

Ouji A, El-Bok S, Mouelhi M, Ben Younes M, Kharrat M (2016) Yield and yield components of Chickpea (*Cicer arietinum* L.) as influenced by supplemental irrigation under semi-arid region of Tunisia. World J Agric Res 4:153–157

Pan Y, Seymour GB, Lu C, Hu Z, Chen X, Chen G (2012) An ethylene response factor (ERF5) promoting adaptation to drought and salt tolerance in tomato. Plant Cell Rep 31:349–360

Pandey P, Irulappan V, Bagavathiannan MV, Senthil-Kumar M (2017) Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. Front Plant Sci. https://doi.org/10.3389/fpls.2017.00537

Puranik S, Sahu PP, Srivastava PS, Prasad M (2012) NAC proteins: regulation and role in stress tolerance. Trends Plant Sci 17:369–381

Ram G, Sharma AD (2013) In silico analysis of putative miRNAs and their target genes in sorghum (*Sorghum bicolor*). Int J Bioinform Res Appl 9:349–364

Redillas MCFR, Park SH, Lee JW, Kim YS, Jeong JS, Jung H, Bang SW, Hahn TR, Kim JK (2012) Accumulation of trehalose increases soluble sugar contents in rice plants conferring tolerance to drought and salt stress. Plant Biotechnol Rep 6:89–96

Rong W, Qi L, Wang A, Ye X, Du L, Liang H, Xin Z, Zhang Z (2014) The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat. Plant Biotechnol J 12:468–479

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C_T method. Nat Protoc 3:1101–1108

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot 2012:1–26

Siddiqui MH, Al-Khaishany MY, Al-Qutami MA, Al-Whaibi MH, Grover A, Ali HM, Al-Wahibi MS, Bukhari NA (2015) Response of different genotypes of faba bean plant to drought stress. Int J Mol Sci 16:10214–10227

Sinha P, Pazhamala LT, Singh VK, Saxena RK, Krishnamurthy L, Azam S, Khan AW, Varshney RK (2016) Identification and validation of selected universal stress protein domain containing drought-responsive genes in pigeonpea (*Cajanus cajan* L.). Front Plant Sci. https://doi.org/10.3389/fpls.2015.01065

Soares-Cavalcanti NM, Belarmino LC, Kido EA, Wanderley-Nogueira AC, Bezerra-Neto JP, Cavalcanti-Lira R, Pandolfi V, Nepomuceno AL, Abdelnoor RV, Nascimento LC, Benko-Iseppon AM (2012) In silico identification of known osmotic stress responsive genes from Arabidopsis in soybean and Medicago. Genet Mol Biol 35:315–321

Song K, Kim HC, Shin S, Kim KH, Moon JC, Kim JY, Lee BM (2017) Transcriptome analysis of flowering time genes under drought stress in maize leaves. Front Plant Sci. https://doi.org/10.3389/fpls.2017.00267

Sourour A, Afef O, Mounir R, Mongi BY (2017) A review: morphological, physiological, biochemical and molecular plant responses to water deficit stress. Inter J Engin Sci 6:1–4

Swapna S, Shylaraj KS (2017) Screening for osmotic stress responses in rice varieties under drought condition. Rice Sci 24:253–263

Torres AM, Avila CM, Gutierrez N, Palomino C, Moreno MT, Cubero JI (2010) Marker-assisted selection in faba bean (*Vicia faba* L.). Field Crop Res 115:243–252

Wu GQ, Wang CM, Su YY, Zhang JJ, Feng RJ, Liang N (2014) Assessment of drought tolerance in seedlings of sugar beet (*Beta vulgaris*

L.) cultivars using inorganic and organic solutes accumulation criteria. Soil Sci Plant Nutr 60:565–576

Wu M, Liu H, Han G, Cai R, Pan F, Xiang Y (2017) A moso bamboo WRKY gene PeWRKY83 confers salinity tolerance in transgenic Arabidopsis plants. Sci Rep 7:11721. https://doi.org/10.1038/s41598-017-10795-z

Xiang Y, Huang Y, Xiong L (2007) Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. Plant Physiol 144:1416–1428

Xie R, Pan X, Zhang J, Ma Y, He S, Zheng Y, Ma Y (2018) Effect of salt-stress on gene expression in citrus roots revealed by RNA-seq. Funct Integr Genom 18:155–173

Xu ZZ, Zhou GS, Wang YL, Han GX, Li YJ (2008) Changes in chlorophyll fluorescence in maize plants with imposed rapid dehydration at different leaf ages. J Plant Growth Regul 27:83–92

Xu R, Yamada M, Fujiyama H (2013) Lipid peroxidation and antioxidative enzymes of two turfgrass species under salinity stress. Pedosphere 23:213–222

Xu Q, He J, Dong J, Hou X, Zhang X (2017) Genomic survey and expression profiling of the MYB gene family in watermelon. Hortic Plant J 4:1–15

Yang A, Dai X, Zhang WH (2012) A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice. J Exp Bot 63:2541–2556

Yang G, Wang Y, Xia D, Gao C, Wang C, Yang C (2014) Overexpression of a GST gene (*ThGSTZ1*) from *Tamarix hispida* improves drought and salinity tolerance by enhancing the ablity to scavenge reacive oxygen species. Plant Cell Tiss Organ Cult 117:99–112

Yang X, Liu J, Xu J, Duan S, Wang Q, Li G, Jin L (2019) Transcriptome profiling reveals effects of drought stress on gene expression in diploid potato genotype P3–198. Int J Mol Sci 20:852. https://doi.org/10.3390/ijms20040852

Zhang H, Liu W, Wan L, Li F, Dai L, Li D, Zhang Z, Huang R (2010) Functional analyses of ethylene response factor JERF3 with the aim of improving tolerance to drought and osmotic stress in transgenic rice. Transgenic Res 19:809–818

Zhang Z, Zhou Q, Yang Z, Jiang J (2013) Discovery AP2/ERF family genes *in silico* in *Medicago truncatula*. Afr J Biotech 12:3636–3642

Zhao J, Sun Z, Zheng J, Guo X, Dong Z, Huai J, Gou M, He J, Jin Y, Wang J, Wang G (2009) Cloning and characterization of a novel CBL-interacting protein kinase from maize. Plant Mol Biol 69:661–674

Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, Wang CM, Wang HW, Zhang JS, Chen SY (2008) Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer diferential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol J 6:486–503

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273