Plant Archives Volume 20 No. 2, 2020 pp. 9669-9681 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

PHYSIOLOGICAL AND MOLECULAR STUDIES ON WILD BARLEY (*HORDEUM SPONTANEUM*) UNDER SALT STRESS

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Abstract

Salinity is a major threat to plant survival and productivity worldwide. Wild barley (*Hordeum spontaneum*) is the progenitor of the cultivated barley (*Hordeum vulgare* L.) and one of the known species for tolerance to excess of salinity in soil and irrigating water. We evaluated some of the physiological and molecular variations resulting from germinating wild barley seeds on different salinity levels. Chlorophyll fluorescence parameters (OJIP, Fv/Fm and PI) were measured in wild barely seedling growing in the presence of ten different NaCl levels (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 mM) for 4 weeks. Chloroplast photosynthesis efficiency begins to be negatively affected at 125 mM NaCl concentration and above. Molecular analysis using DDRT-PCR technique on wild barley seedlings growing at 0, 125 and 200 mM NaCl levels, revealed the upregulation of several fragments. Sequencing revealed that the fragments belongs to seven genes (thi4, shmt, lhc3, B2, vps29, oee1 and sbt). Real-time PCR analysis was performed to validate our results and out of the seven identified genes, B2 was the most upregulated gene followed by shmt and oee1 genes.

Key words: *H. spontaneum*, salinity, chlorophyll fluorescence, DDRT-PCR and real-time PCR analysis

Introduction

Dehydration-inducing conditions are major abiotic stresses affecting plant distribution, productivity, and survival worldwide (Hernández, 2019). Salinity is a limiting factor affecting plants' growth and yield in different areas worldwide (Munns, 2005; Munns and Tester, 2008 and Hasegawa, 2013). Soil salinization occurs naturally in areas close to/surrounding see-shores and salty lakes, or due to wrong agricultural practices, usage of poor-quality irrigation water, lack of proper drainage system, and extensive use of fertilizers (FAO, 2009). Salinization of soil is a problem in different regions worldwide, and salinization of lands is estimated to reach up to 50% of the world's arable land by 2050 (Wang *et al.*, 2003; Jamil *et al.*, 2011 and Shrivastava and Kumar, 2015), therefore hindering efforts towards increasing food production for the growing world population.

H. spontaneum is commonly known as wild barley or spontaneous barley. It is originated in the fertile crescent area (Turuspekov *et al.*, 2014), and believed to be the origin that gave rise to commercially available cereal barley (*H. vulgare* L.) (El-Hashash and El-Absy, 2019). Economically important crops-wild relatives are the genetic source for various traits involved in biotic and abiotic tolerance (Mondal *et al.*, 2018 and Pandotra *et al.*, 2020). The domestication process of many commercial crops led to the marked truncation of the wild population, leading to loss of the genetic pool available to breeders (Ellis *et al.*, 2000 and Kumar *et al.*, 2020). Therefore, different workers in the field of plant biology have used wild barley for the discovery of new genes (Ames *et al.*, 2015; Nishantha *et al.*, 2018 and Liu *et al.*, 2020a).

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The differential-display reverse transcription-PCR (DDRT-PCR) technique has been extensively used to detect genes differentially expressed under different stress conditions (Lee *et al.*, 2019 and Zhang *et al.* 2020). Also, this technique has been applied to detect salinity-induced genes in barley (Muramoto *et al.*, 1999; Wei *et al.*, 2001 and Kadri *et al.*, 2014).

The principle underlying using chlorophyll fluorescence to screen different genotypes against osmotic-inducing stress conditions is well documented (Jedmowski *et al.*, 2015). In cereals, chlorophyll fluorescence has been used with a great degree of success to differentiate between environmentally sensitive/tolerant genotypes (Sayed, 2003; Paknejad *et al.*, 2007; Kalaji and Guo, 2008; El-Hendawy *et al.*, 2019 and Ebrahim *et al.* 2019).

The current work is focus on identifying alteration at gene transcript levels in wild barley using chlorophyll fluorescence parameters as a pre-screening step and followed by differential display technique and qPCR. The prescreening technique described here within could be of benefit to plant biologists working on salinity. The easiness and reproducibility of the method described here with the new sets of primers provided within serves as a tool to analyze the transcriptional alteration of plants in less advanced/well-equipped scientific institutes.

Materials and Methods

2.1 Plant materials

Max Planck Institute, Germany, kindly donated seeds of wild barley (*H. spontaneum*) "collected from the northern coastal region of Egypt Wadi Habies."

2.2 Salinity experiment on wild barley *H. spontaneum*

Seeds were germinated on filter paper treated with different salinity levels (0, 25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 mM NaCl). Five days post-germination, the seeds were transplanted into pots filled with peat moss (3 seeds/pot, four pots/treat; each pot is considered as a single biological replicate). The plants were irrigated every 5-6 days with the corresponding NaCl solution. The plants were kept in growth chambers under the following conditions: 16/8 h light/dark photoperiod at 400 µmolm-2s-1light intensity; humidity was adjusted at 70%, and the temperature was adjusted at 26 and 24^{0}C during light/dark cycles, respectively.

2.2.1 Measurements of chlorophyll fluorescence

Using a Plant Efficiency Analyzer (Pocket-PEA, Hansatech, UK), chlorophyll fluorescence measurements were recorded 24 days post-transplanting into the soil. The second leaf from the bottom of plants was used to perform the measurement, following the procedure of Strasser *et al.* (2000, 2004). Measurements were taken for ten plants for each treatment at the end of the dark hours, in which weak dim green light from a lamp was used for orientation. A saturating light pulse of 3,500 µmol m^{-2} s^{-1} with a peak wavelength of 627 nm was used to illuminate plants (using a single light-emitting diode for 1 sec). The collected data were processed using the PEA-Plus program 1.10. (Copy right©, 2011). The induction curves were analyzed according to the OJIP-test protocol, Fv/Fm, and performing index (PI) were analyzed, too (Strasser *et al.* 2000 and 2004).

2.2.2 RNA extraction

RNA extraction was performed on plants derived from the following treatments 0, 125, and 200 mM NaCl. The second leaf of each pot was collected and pooled together. Plant tissues grounded in liquid nitrogen with mortar and pestle and a 100 mg of the tissues were further grinded by placing in 2ml centrifuge tubes containing two 5mm diameter stainless steel balls, using mixer mill MM400 (Retsch-Haan, Germany) at a 25rpms^{-1} frequency for 30s. RNA extraction was performed using the TriFastTM extraction kit (Cat no. 302020, PeqLab, Germany). RNA samples were measured using the NanoDrop-2000 spectrophotometer (Thermo-Fisher Sci., Germany).

2.2.3 cDNA synthesis

15µg of RNA samples were incubated with ten units of DNase (Cat no. EN0521, Thermoscientific™) at 37°C for 30 min for removal of any DNA contamination. RNA was re-concentrated again using Concentrator™-25 kit (Cat no. R1018, Zymoresearch™) and measured again using Nanodrop. 5 µg of the resulted RNA was used for cDNA synthesis using the Maxima H-RT kit (cat # EP0751, Thermoscientific™). Following a 60 min incubation at 42°C, the resulted cDNA was diluted two folds.

2.2.4 DDRT-PCR

Fourteen arbitrary primers were used (Yang *et al.*, 2006, Table 1a). The procedure was conducted by mixing the following components {4 µl cDNA +10 µl PCR 10X buffer +1 µl oligo dT (GGCGCGCCT18) (10 pmol) +1 µl Arbitrary primer (10 pmol) + 1 µl dNTPs (10 mM) + 1 µl Phire HotStart II polymerase enzyme (Cat # F122S, Thermoscientific™)} for any given reaction. The final volume was adjusted to 50 ml using ddH2O. PCR amplification conditions used: 98 °C for 1min, followed by one cycle of (98°C for 15 sec, 20°C for 30 sec, 72°C for 30 sec) and then 35 cycles of (98°C for 15 sec, 48°C

for 15 sec, 72°C for 30 sec), and finally 72°C for 60 sec. 5 µl of PCR product was used to check for up/down regulated bands on 1.5% agarose gel.

2.2.5 Bands excision, purification, and cloning

For differentially expressed bands, 45 ml of the PCR product was loaded in 3% agarose gel for band excision; DNA fragments were gel-extracted (GenJet kit, Cat no. K0831, Thermoscientific™). Eluted fragments were cloned into pJET1.2 vector for sequencing using Clone JET PCR kit (Cat no. K1231, Thermoscientific™). The resulted sequences were blasted against previously published data at https://www.ncbi.nlm.nih.gov/.

2.2.6 Real-time PCR

New primers were designed for further analysis of deferentially isolated fragments using real-time PCR (Table 1b). RNA extraction and cDNA preparation were conducted only for wild barley plants growing at 0, 50, 100, 150, 200, and 250 mM NaCl. The resulting cDNAs were diluted 4 times with ddH_2O, Quanti Nova™ SYBR Green PCR kit (Cat no. 208052, Qiagen™) was used to perform a real-time PCR reaction. In a fresh real-time PCR MicroAmp™ plates (cat no. N8010560, Thermoscientific™), the following components were added: 10 µl Quanti Nova™ SYBR mix +2 µl cDNA +0.1µl Rox dye +0.3 µl Forward/Reverse primer (5pmol) + adjusted to 20 ml using ddH2O. The real-time PCR machine (Biometra™, Germany) was adjusted to the following conditions: 95°C for 2 min, followed by 39 cycles of (95°C for 30 sec, 60°C for 20 sec). The PCR reactions were performed for all the identified genes and tubulin "as reference gene" to detect levels of gene expression at two concentrations of salt (0, 200 mM NaCl), selected genes were further tested with the rest of salt levels (0, 50, 100, 150, 200, 250 mM NaCl). In general, three biological replica were tested for every reaction for each gene. Calculation of relative fold change of gene expression was performed using $2^{-}\Delta\Delta CT$method {ΔΔCT= (Cttarget – Ct ref) treated – (Cttarget – Ct ref)untreated} (Livak and Schmittgen, 2001).

2.3 Statistical analysis

The chlorophyll fluorescence obtained data were analyzed using the GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, USA).Shapiro-Wilk test was used to test the normality of data. Analysis of variance (ANOVA) followed by multiple comparison tests to test the significance of data and compare different means. Significant values at $p< 0.1$, 0.05 and 0.01 levels are marked with *, **, and ***, respectively. WASP-Web Agri Stat Package 2.0 was used to analyze real-time PCR data. ANOVA followed by the least significant test was used to test the significance of data and separate means at critical difference 0.05. Values marked with the same letter are not significantly different.

Table 1: List of primers used in the analysis of wild barley.

ID	Sequence (5'-3')	length
a. Differential display arbitrary primers		
arb **1**	GGCGCGCCGATTCCTC	16
arb **2**	GGCGCGCCGTTTCCTC	16
arb **3**	GGCGCGCCTCTAGGAG	16
arb **4**	GGCGCGCCAAGAGCGA	16
arb **5**	GGCGCGCCTACAGCAG	16
arb **6**	GGCGCGCCTCATGGCT	16
arb **7**	GGCGCGCCTCTTGGGT	16
arb **8**	GGCGCGCCCTTTGGTC	16
arb **9**	GGCGCGCCCTCACAAC	16
arb **10**	GGCGGGCGCGCCGTGACATG	16
arb **11**	GGCGCGCCTACTCCCT	16
arb **12**	GGCGCGCCATCTCCGA	16
arb **13**	GGCGCGCCTCTTCCGA	16
arb **14**	GGCGCGCCTCATCCGA	16
b.Designed primers for real-time PCR reactions		
thi4F	GACGCTCAAGAACGTTACCC	20
thi4R	GGGCAGTGACATGAACAAAA	20
B2F	GGGAGGACAAGAAGTGCAAC	20
B2R	ATCAGCACTGAGCAGCCTTC	20
vacF	GGCCTCCGTGTTGTTGTCTA	20
vacR	TCCATATCGAGCAGGAGGGT	20
cabF	GACGACCCTGTTACCTTCGC	20
cabR	GCTATTGCACACACATGGGA	20
serF	GACCTCGAGAGGTTTGGTCG	20
serR	ATGTCTGACACCAGGAAGCC	20
oeeF	TCGACGAAATCCAGAGCAAGA	21
oeeR	GCCTTCCATCTCGTCAAGGG	20
subF	CGGCGTCCATGTAGAGAGAAT	21
subR	ATTCCAGAGATTAATGGAGTAACCTTC	27
tubF	TTACCTCACCGCCTCTGCCA	20
tubR	CCAATGCAAGAAAGCCTTCC	20

Results and discussion

3.1 Salinity experiment on wild barley (*H. spontaneum*)

Wild barley seeds were germinated and grown on ten different concentrations of NaCl (0, 25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 mM NaCl) for 4 weeks (Fig.1a). To differentiate between wild barley performance on the different treatments, chlorophyll fluorescence was measured, and OJIP induction graphs were drawn for each concentration compared to the non-salt treated control (O at 0 ms, J at 2 ms, I at 30 ms and P at 300 ms, Banks, 2017). No differences in fluorescence

were observed in OJIP graphs in plants growing at 25, 50, 75 mM NaCl concentrations, compared to the control. At 100 mM NaCl JI stage was slightly raised, while the rest of graph stages were raised at 125 mM NaCl or higher. The increase in fluorescence was clearer, with concentrations above 125 mM and raised up to 200 mM NaCl (Fig. 1b). Further analysis using performing index PI and and Fv/Fm was performed; PI essentially an indicator of sample vitality and it represents an internal force of the sample to resist constraints from outside (Kalaji and Guo, 2008); the Fv/Fm is representing the maximum quantum efficiency of Photosystem II (Kalaji and Guo, 2008).

For PI, measured values at low salt concentrations of 25, 50, 75 and 100 mM NaCl (11.57, 11.64, 11.23 and 11.44, respectively) showed higher level than the control (10.54). At 125mM NaCl slight decrease in PI values was detected (10.49). High significant difference for salt treated plants versus control was recorded at 200mM and the following higher concentrations 225 and 250 mM NaCl (6.984, 7.371 and 7.27, respectively) (Fig. 1c). For Fv/Fm, recorded value at low salt concentration of 25 mM NaCl (0.8403) showed higher level than the control (0.8372). At 50, 75 and 100 mM NaCl Fv/Fm values were similar to the control. At 125 mM NaCl level, slight decrease in Fv/Fm values were detected (0.8326). High significant difference for salt treated plants versus control were recorded at 200, 225, and 250 mM NaCl (0.8262,

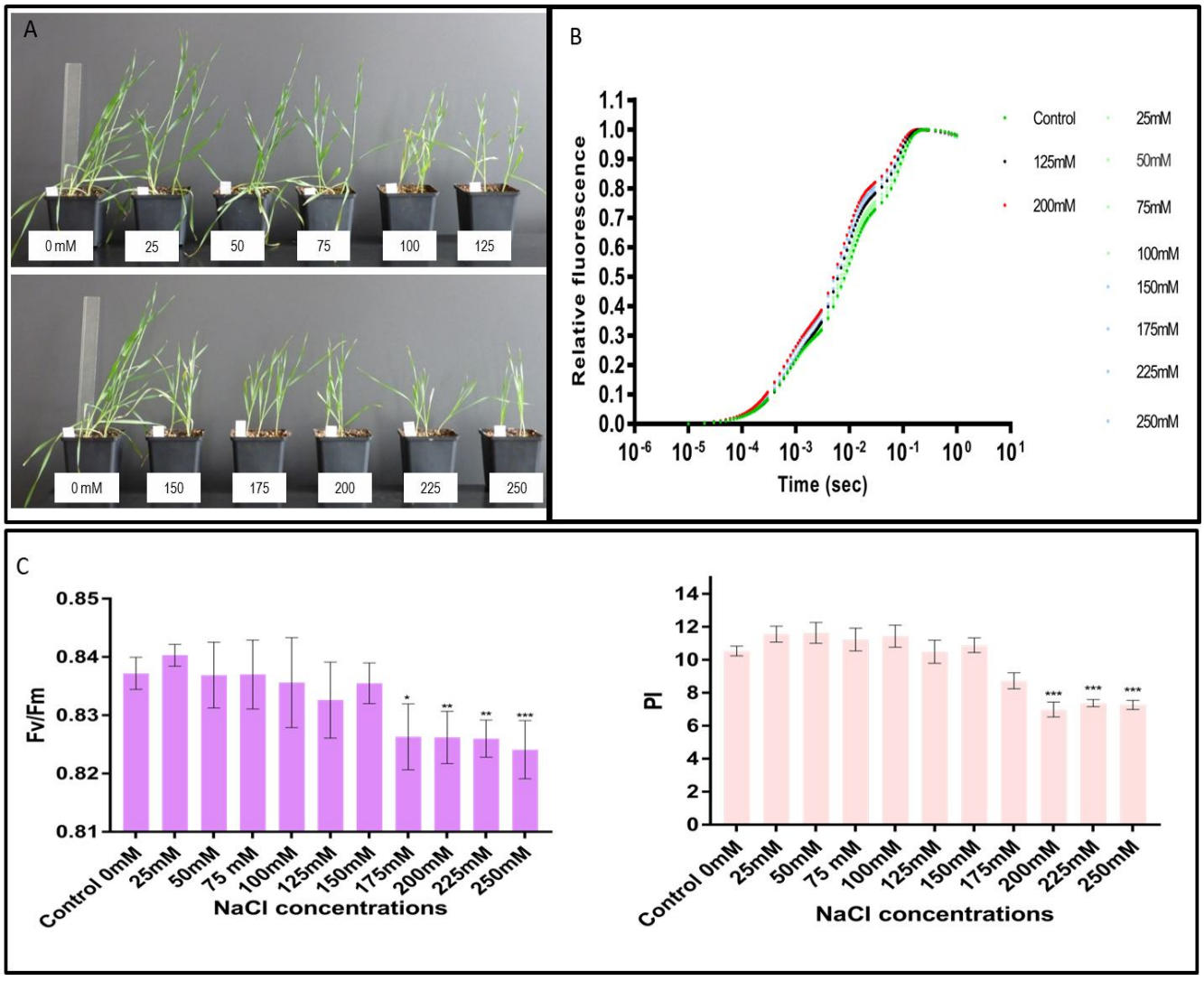

Fig. 1: A)Wild barley *(Horduem spontaneum)* under different NaCl concentrations. B) OJIP graphs of chlorophyll fluorescence under different NaCl concentrations. C) Fv/Fm and PI performance index graphs under different salinity concentrations, all datasets shown are presented as means ± standard error (SE). For Fv/Fm one-way ANOVA Friedman non-parametric test followed by Dunn's multiple comparisons tests were used to assess the significance of differences between different treatments and control at $P< 0.05$. For PI one-way ANOVA followed by Dunnett's multiple comparisons tests were used to assess the significance of differences between different treatments and control at $P< 0.05$

0.826 and 0.8241, respectively) (Fig. 1c). Although some Fv/Fm and PI values were higher in salt-stressed plants under low salt concentrations than controls, these measurements were statistically insignificant. Yet, statistically significant decreases in Fv/Fm and PI values were only recorded at a salt concentration above 175 mM NaCl. These results are in line with the results reported by Koyro *et al*. (2013). Koyro *et al*. (2013) found that when growing Panicum turgidum plant (halophytic plant) in a low salt medium (125 mM NaCl), fresh and dry biomass and Fv/Fm were similar to their counterparts under non-stressed conditions. Kalaji *et al*. (2011) exposed barley cultivars to 120 mM NaCl for 24 hours and found no significant decrease in Fv/Fm values. Nevertheless, longer exposure (7 days) caused a significant decrease in Fv/Fm values in sensitive cultivars compared to a slight decrease in tolerant cultivars, while Abdeshahian *et al*. (2010) concluded that low concentrations of NaCl (110 and 165 mM NaCl) caused no significant decrease in Fv/Fm values in wheat.

3.2 Performing DDRT-PCR technique and analysis of results in wild barley

Based up on chlorophyll fluorescence results, wild barley plants growing on the following NaCl concentrations (0, 125- immediate stress and 200- high stress mM NaCl) were selected for the DDRT-PCR study. The amplifications were performed using 14 arbitrary primers and exactly 11 Up-regulated bands were recovered from the agarose gel, cloned in the pJET 1.2/blunt vector and sequenced successfully. Sokolov and Prockop (1994) tested the DDRT-PCR original procedures of Liang and Pardee (1992) and of Welsh *et al*. (1992). They clamid that although the methods were useful, they encountered several limitations. (a) Because the DNA fragments were separated on a DNA sequencing gel, the procedures were limited to fragments of less than about 600 bp. (b) The initially isolated PCR products had to be re-amplified once or twice in order to generate adequate amounts for Northern blot assays, cloning, sequencing, etc.

Liu *et al.*, (2020b) employed transcriptome differential expression analysis of Prunellae Spica under salt stress. RNA-seq analysis was performed for Prunellae Spica treated with NaCl at different concentrations (0, 50, 150 and 200 mM). A total of 3857 genes were differentially expressed under low, medium and high salt stress, including 2456 up- regulated and 1401 down-regulated differentially expressed genes (DEGs). To test the quality of the differential expression analysis, 10 DEGs associated with salt stress response processes were randomly selected for qRT-PCR verification, and the results were compared with those of the RNA-seq. Out of the 10 DEGs, 3 DEGs had the maximum expression at 150 mMNaCl then the expression declined at 200 mMNaCl. This pattern of expression was similar to our study. On the other hand, 2 DEGs expression gradually increased with increasing salt concentration. The rest of the DEGs were inhibited more severely under medium NaCl treatment than under low or high NaCl treatment.

Moreover, they found that re-amplification did not succeed for many fragments and re-amplification by PCR frequently generated multiple bands. (c) The procedures of elution of DNA fragments from DNA sequencing gels, re-amplification, verification and re-purification of DNA fragments required use of radioisotopes and were relatively time consuming. For these reasons, they

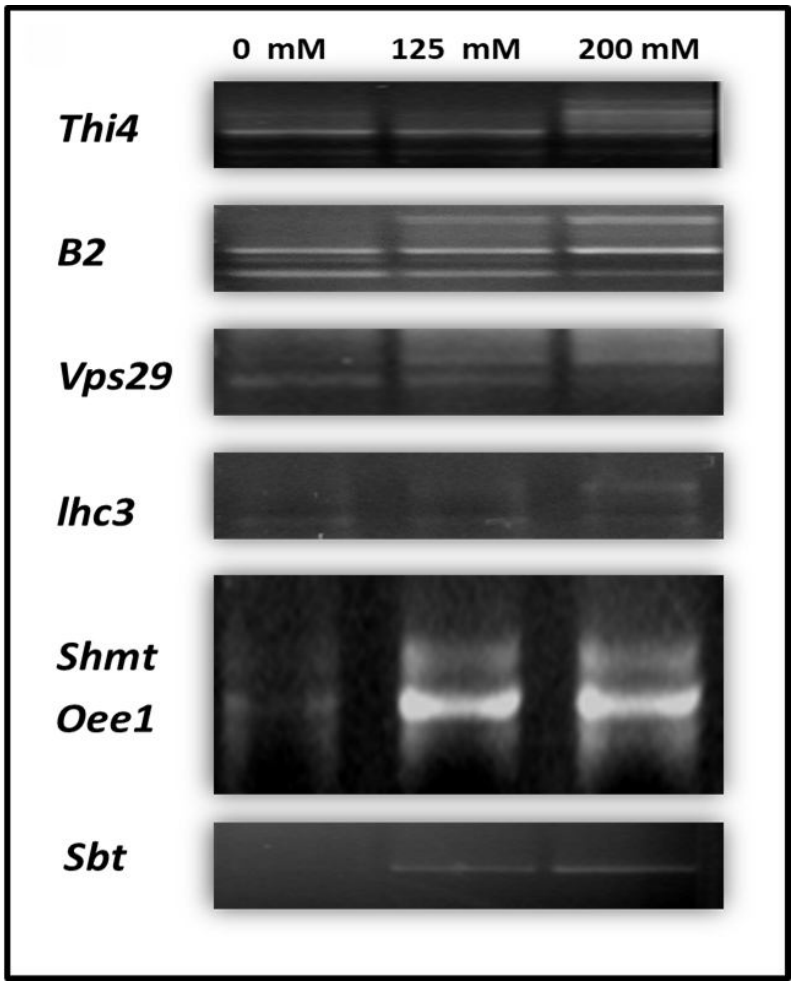

Fig. 2: Differential gene expression on wild barley under 0, 125 and 200 mM NaCl detected on agarose gel.

Table 2: List of sequences of the wild barley identified fragments.

arb primer5'-3'	Gene	Sequence (5'-3')
GGCGCGCC GATTCCTC	*thi4*	attcctcgacgagctcaacatcgagtacgacgagcaggaggactacgtcgtcatcaagcacgccgcgctcttcacctccaccatcatg agccgcctcctcgcgcgccccaacgtcaagctcttcaacgccgtcgccgtggaggacctcatcgtcaaggagaaccgcgtcgccgg agtcgtcaccaactgggcactcgtccccatgaaccacgacacacagtcctgcatggaccccaacgtcatggaggccaaggtcgtggt gagctcctgcggccacgacgggcccttcggcgccaccggggtcaagcggctccaggacatcggcatgatccaggcggtgcccgg gatgaaggcgcttgacatgaacacggccgaggacgccwtcgtgcgcctcacccgcgaggtcgtcccgggcatgatttgtcaccgg catggaggtygccgagatcgacggtgccccgagaatgggcccgaccttyggcgccatgatgatctccggccagaaggcggcgca cctggcgctcaaggccctcggccggccgaacggcatygacgggacgctcaagaacgttaccccggcgctgcacccggagatgat cctggcggcgaccaacaacgccgacatygtygacgcctaagcaaccagctggtgtttgggggcaacaaatcaataagatggttcg gtgaaacagaggatgcttagggacgagggcttgcctatgtgatttgtcagamtygtgtttaattccgatgttatctttagttttyttatgtt agtgtattttgttcatgtcactgcccgccatgtgctytgctytgctytgccccatgtcagctccatggatgatgagtccttytgtaacma grgggaataaaaaggttgaatytatgaccctgattggaaaaaaaaaaaaaaaaaa
GGCGCGCC AAGAGCGA	*b2*	gagaggggacaacaaccacggcgccgcctcggagaagcggttcaagacgctgccggcgtccgaggcgcttcctaggaacgagcc catcggcggctacatcttcgtctgcaacaacgacaccatggaggagaacctcaagaggcagctcttcgggttgccatccagatacaga gattcagtgagggcgatcaggccggggctgcccctcttcctctacaactactccacccaccagctccacggcatcttcgaggccgcga gctttggcggagcaaacatcgacccggcggcgtgggaggacaagaagtgcaacggcgagtcccgcttccctgcacaggtgaggg tggccacaaggaagatctgcgacccactggaggaggacgcattccgccctatcctgcaccactacgacgggcccaagttccggctg gagctcagcatcaccgaggctctctcccttcttgatatatttgaagacaaggatgatgcatgatgaaggctgctcagtgctgatcaggg gactgttgaggaaggaacagcgtgcaataagatcgaccggagtatacatacatacatacgatttatatcattatcaattaatggtgatatat aagtgagctttattgggttttccatctcacgggctatgaaattatgcsccccccgttgtctgtaatgcgtggtgatggctgcagaagatga gccytcgtgaacataacttatgtactgtcacggctatgtatgtataatactattgagactttaagatgtttctcctgttgtacmccttataaca gaataaatcatggccttcatgtttgccaaaaaaaaaaaaaaaaa
GGCGCGCC TACAGCAG	*Vps29*	catcacatatgacgtcaacccgagcttcgtgctcatggacattgatggcctccgtgttgttgtctacgtgtacgagctcattgacggcgag gtgaaggttgacaagatcgacttcaagaagaccgccacgatgcatggctgatggcgctttccacatgacagaaatccgatcctgtttgtt cttatttttgcgcccccaccttccagagctcttttgttttgtttcccgtactagttgtaccctcctgctcgatatggaaaaccagagctgtgttta catattaatgaatgtgattactcaaaaaaaaaaaaaaaaaa
GGCGCGCC TCTTGGGT	*lhc3*	caattgtagagwcttctagaagatggcgcgcctcttgggtgcgtcttccccgaggtgctccagaagtgggtgggcgtcgagttcaagg agcccgtctggttcaaggccggctcgcagatcttctctgagggtggtctcgattaccttgggaaccccaaccttgtccacgcgcagagc atccttgccgtgcttggcttccasgtccttctcatgggtcttgttgaargttccgcatcaacggtctcgatggtgtyggcgagggcaacg acctytaccccggcggccagtacttcgacccgctyggccttgccgacgaccctgttaccttcgcggagcttaaggtgaaggagatcaa gaatggccgtcttgccatgttytytatgttcgggttyttcgtgcaagctatygtcaccggcaagggcccattggagaacctgttygaccm cctygacgaccctgtcgccaataatgcatgggttttcgccmccaaatttgsgcctggatyttaggctgcccgacgsgccmcggtggcc tgccattgtaaaatgtaaattattcccatgtgtgtgcaatagcaacgaatggtcgatgtgatccctgaaaaaaaaaaaaaaaaa
GGCGCGCC CTCACAAC	*shmt*	caccagattgctgccctggcagttggcctgaagcargccatgttacctggattcaargcatatattcagcaggtcaaggtcaatgctgttg cccttggaaaccatctcatgagtaagggctacaagttagttactgatggaacagagaaccaccttgttctctgggatcttcgccctcttgg cttgtctggtaacaaagttgagaaagtatgtgatctgagcagcattacactcaacaaaaatgctgtctttggtgacagcagtgcattggca cctggtggtgtccgaattggtacacctgccatgacctcgagaggtttggtcgagaaggacttcgtgaagattgctgagtaccttcatcag gctgtggtcatctgcttgaatgtccagaagcagcgaggcaagcgttacaatgatttcattgttgacttggagaagaacgaggacatagc ggagctcagggcagaggtcgagaagtttgccatttcatttgagatgcctggcttcctggtgtcagacatgaaatacaaggattaggcaa tcgttttyttgtagcagaaaattggtgccttaaggggstgattgctgtgcgctaatcgtgatgtatggattatggttgagaatgatatgactc atgaataatcgttgaaggtatgcgatgaaaaaaaaaaaaaaaaa
GGCGCGCC CTCACAAC	*oee1*	ctctgccctcctcgtctctggcgcaagtgcggaggggcgcccaagaggctgaccttcgacgaaatccagagcaagacctacatgga ggtgaagggtaccggcaccgcgaaccagtgcccgaccatcgacggcggcgtcgactccttccccttcaaggccggcaagtacgaga tgaggaagttctgcctggagcccacctccttcaccgtcaaggccgagggcatccagaagaacgagccaccggccttccagaagacca agctcatgacccgtctcacttacacccttgacgagatggaaggcccgctcgaggwcsgcgccgacggcrccctcgagttcsaggaga aggattgctcgactacktcgccggctgtggtcacccgcctgaatgtcsagargcasckasgccagcgttacaatgatttcmttgttgactt ggagaagarmraggacatagcggagctcacggcagakgtckagaagtttgccatttcatttgakatgcctggcttcctggtgtcagaca tgamatacmmcgattasgcwatcgttttcttgtarcasaaaattggtgccttmaggggctgattgctrtgcgcyatcgtgatgtwtggat tatggttsa
GGCGCGCC GTGACATG	*sbt*	tggccggagagcgccgaggcggcgtccatgtagagagatgtagcgacgctcgtgtcacacgattgtttgagtttactctgcgcgcga gcctgtgaaggttactccattaactctggaataatttatcttcttggatcataataataatacgatctcgtactcaaaaaaaaaaaaaaaaaa

developed an agarose gel electrophoresis DDRT-PCR. DNA bands up to 3000 bp were readily isolated by agarose gel electrophoresis and the yields of the products were sufficient so that DNA bands of interest could be used directly for Northern blot analysis, cloning and nucleotide sequencing. Also, in this respect, a modified, nonradioactive differential display technique was described by Lohmann *et al.* (1995), who used silver staining for the detection of differentially expressed bands. However, following their protocol, Jung *et al.*, (1997) could not obtain a clear banding pattern for differentially expressed bands. Moreover, re-amplification of silver-stained products often revealed many bands, and several rounds of purification were necessary to obtain a single product. Therefore, Jung *et al.* (1997) used 1.6% ethidium bromide (EtdBr)-stained agarose gels for detection of differentially displayed RT-PCR products successfully. Later on, in agreement with our results, different authors used agarose gel in DDRT-PCR analysis as Liu *et al.*, (2007), Sireesha *et al.*, (2017) and Abdel-fattah *et al.*, (2020). On the otherhand others used polyacrylamide gel in the analysis of DDRT- PCR

Table 3: Sequence analysis of identified fragments from differential display of wild barley under salt stress

Fragment	Crop	Length (bp)	Query cover	E-Value	Per. Ident.
1st		920			
Unknown mRNA	*H. vulgare*		96%	0.0	97.7%
Unknown mRNA	*T. aestivum*		95%	0.0	96.57%
Predicted: Thiamine thiazole synthase, chloroplastic, mRNA	*A. tauschii*		69%	0.0	96.26%
2nd		840			
Unknown mRNA	*H. vulgare*		75%	0.0	99.2%
Unknown mRNA Predicted: B2-protein mRNA	*T. aestivum* *A. tauschii*		99% 92%	0.0 0.0	89.84% 91.06%
3rd		318			
Unknown mRNA	*H. vulgare*		100%	5e-160	100.00%
Predicted: vacuolar protein sorting-associated protein 29 mRNA	*A. tauschii*		59%	5e-76	95.68%
Unknown mRNA	*T. aestivum*		55%	8e-69	95.35%
4th		618			
type III LHCII CAB precursor protein mRNA	*H. vulgare*		93%	0.0	96.35%
Unknown mRNA	*T. aestivum*		92%	0.0	92.99%
Predicted: chlorophyll a-b binding protein of LHCII type III mRNA	*A. tauschii*		93%	0.0	91.75%
5th		679			
Unknown mRNA	*H. vulgare*		97%	0.0	98.79%
Predicted: Serine hydroxymethyl transferase mRNA	*A. tauschii*		96%	0.0	93.75%
Unknown mRNA	*T. aestivum*		92%	0.0	93.81%
6th		626			
Unknown mRNA	*H. vulgare*		57%	9e-176	97.53%
Predicted: oxygen-evolving enhancer protein 1 mRNA	*A. tauschii*		57%	9e-166	95.88%
oxygen evolving enhancer protein mRNA	*T. aestivum*		57%	3e-165	95.87%
7th		180			
Unknown mRNA	*H. vulgare*		87%	2e-70	98.73%
Predicted subtilisin-like protease mRNA	*A. tauschii*		91%	2e-37	82.39%
subtilisin protease mRNA	*T. aestivum*		67%	3e-23	81.06%

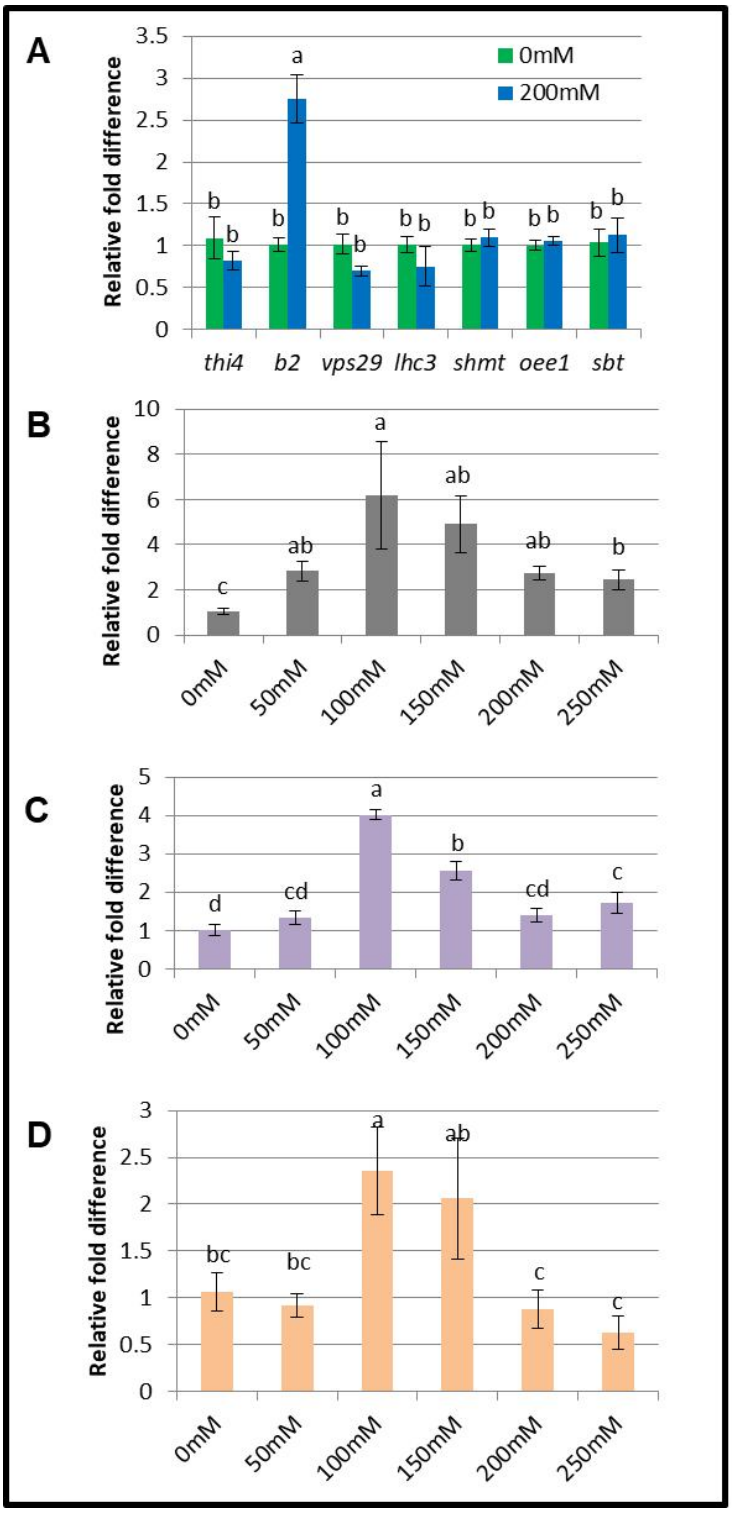

Fig. 3: Real-time PCR for the identified genes under different salinity levels. A) changes in relative fold difference in gene expression for thi4, b2, vps29, lhc3, shmt and sbt fragments under 0 and 200 mM NaCl. Changes in expression levels at different levels of salinity (0, 50, 100, 150, 200 and 250 mM NaCl) for B2, Shmt and Oee1 genes (B, C and D, respectively). Least significant difference used for comparing means at critical difference of 0.05.

products like Kadri *et al.*, (2014) and Zhang *et al.*, (2020).

Sequencing analysis revealed that seven up-regulated fragments (Fig. 2) (Table 2) had similarities to known gene sequences in the database https://blast.ncbi.nlm.nih.gov/blastn as listed in details in (Table 3).

3.3. Functional annotation of differentially expressed genes

These fragments had homology with annotated sequences in the protein database https://blast.ncbi.nlm.nih.gov/blastx as follow: The 1st fragment showed homology with thiazole biosynthesis enzyme, chloroplastic (thi4) with two matches. First match had 89%, 88% and 87% identity; query cover 68%, 68% and 68%; E-value= 7e-93, 2e-92 and 1e-91 and second match had 97%, 97% and 97% identity and E-value= 4e-90, 7e-90 and 7e-90 in *Hordeum vulgare*, Aegilops tauschii and Triticum urartu respectively. Thiamine (vitamin B1) consists of a pyrimidine moiety and a thiazole moiety. The two halves of the molecule, 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl) -4-methylthiazole, are synthesized by separate pathways (Zurlinden and schweingruber, 1994). Thi4 is involved in biosynthesis of the thiazole moiety. It catalyzes the conversion of NAD and glycine to adenosine diphosphate 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid (ADT), an adenylated thiazole intermediate (Chatterjee *et al.*, 2012).

The 2nd fragment showed homology with B2-protein which contains development cell death domain (DCD). It had 100%, 99% and 96% identity; query cover 58%, 57% and 76%; E-value= 9e-115, 4e-113 and 8e-103 in *Hordeum vulgare*, Aegilops tauschii and Brachypodium distachyon, respectively. B2- protein had a DCD plant specific domain which is shared by several proteins in the plant kingdom. These proteins tend to have some regulatory roles in phytohormone response, embryo development and programmed cell death by pathogens attack or ozone (Tenhaken *et al.*, 2005).

The 3rd fragment showed similarity with vacuolar protein sorting 29 (vps29). It had 100%, 100% and 100% identity; query cover 43%, 43% and 43%; E-value= 2e-21, 2e-21and 2e-21in Zea mays, Aegilops tauschii and Brachypodium

distachyon, respectively. The vps29 is a subunit of the retromer complex which is responsible for the retrieval of mannose-6-phosphate receptors (MPRs) from the endosomes for retrograde transport back to the Golgi (Seaman *et al.*, 1998).

The 4th fragment showed homology with chlorophyll a-b binding protein of LHC^{22} type ^{222} (lhc3). It had 96.18%, 94.90% and 96.18% identity; query cover 76%, 76% and 76%; E-value= 4e-90, 7e-90 and 7e-90 in Aegilops tauschii, Brachypodium distachyon and Triticum urartu, respectively. It is part of proteins that are responsible for collecting and transferring light energy to the reaction centers of PSII (Brandt *et al.*, 1992 and Andersson *et al.*, 2003).

The 5th fragment showed homology with serine hydroxymethyl transferase (shmt). It had 97.19%, 90.45% and 84.27% identity; query cover 78%, 78% and 78%; E-value= 2e-119, 1e-111 and 3e-101 in Aegilops tauschii, Brachypodium distachyon and Oryza sativa Japonica Group, respectively. shmt is a member of the á-class of pyridoxal phosphate dependent enzymes, catalyzes the reversible conversion of serine to glycine and tetrahydrofolate which is critical in the biosynthesis of purine, thymidine, choline and methionine (Trivedi *et al.*, 2002).

The 6th fragment showed homology with oxygen-evolving enhancer protein 1 (oee1). It had 94.87%, 94.87% and 94.02% identity; query cover 56%, 56% and 56%; E-value= 4e-72, 4e-72and 4e-71 in Aegilops tauschii, Leymus chinensis and *Hordeum vulgare*, respectively. The Oee1 plays a crucial role in water-splitting reaction and evolving of oxygen in PSII (meadows *et al.*, 1991; Mayfield 1991; Murakami *et al.*, 2002).

The 7th fragment, as mentioned before in sequence analysis, showed homology with subtilisin-like protease predicted mRNA in Aegilops tauschii and subtilisin protease mRNA in Triticum aestivum. For protein database analysis no significant similarity found for this fragment. Expected proteins from *A. tauschii* and *T. aestivum* mRNAs are serine proteases which have a role in plant development, signaling cascades and also involved in pathogen recognition and immune priming (Figueiredo *et al.*, 2014).

Arefian *et al.*, (2019) conducted a proteomic study on chickpea under salinity stress they found that salinity changes the integrity and functionality of chloroplasts, which in turn impacts cell function as a whole. About 39% of identified proteins are chlorophyll a-b binding proteins, oxygen-evolving enhancer protein, ATP synthase, RuBisCO subunits, carbonic anhydrase, and fructose-bisphosphate aldolase. Two genes (lhc3, oee1) out of the seven detected are related to maintaining chloroplast function. Moreover, thi4 gene as mentioned previously in (Table 3.) had a homology to thi chloroplastic gene. Furthermore, different studies confirmed the role of compatible solutes in mitigating the damage of abiotic stress (Khan *et al.*, 2015 and Kumari *et al.*, 2019) and in the present work two genes (thi4, shmt) are thought to be involved in different compatible solutes production pathways. According to Reyes *et al.*, (2011), eukaryotic cells contain an elaborated network of endomembrane compartments that are interconnected with each other by continuous trafficking of membrane vesicles. Vps29 is essential for pre-vacuolar compartments morphology and normal trafficking in plasma membrane in plants (Nodzyñski *et al.*, 2013). Moreover, Zhu *et al.*, (2002) claimed that correcting protein routes between intracellular compartments is important for many processes in plants, such as abiotic stress tolerance. Chauhan *et al.*, (2011) identified B2 gene as a heat shock protein in wheat plants with an extra character over the known heat shock proteins, its expression was found in the developing seed tissue of wheat. Tenhaken *et al.*, (2005) claimed that this gene is induced by several stress conditions including ozone, osmotic and cold stress. In our study we identified B2 gene under salinity stress conditions. Figueiredo *et al.*, (2014) suggested that several subtilases are specifically induced following pathogen infection and very recently an arabidopsis subtilase (SBT3.3) was hypothesized to function as a receptor located in the plasma membrane activating downstream immune signaling processes. We also identified sbt gene under salt stress which is not noticed before in other studies, so further study is needed concerning sbt role under salt stress.

3.4. Analysis of the expression of the deferentially identified *H. spontaneum* sequences via Real-time PCR under different salinity levels.

Further analysis with real-time PCR was conducted for Wild-type barley plants growing at 0, 50, 100, 150, 200 and 250 mM NaCl were used in this study. At the beginning, Real-time PCR was conducted on all identified genes with tubulin (reference gene) to detect levels of gene expression at two concentrations of salt (0, 200 mM NaCl). Resulted data were analyzed using $2^{-}\Delta\Delta CT$ method according to Livak and Schmittgen, (2001) and illustrated as bar graphs as shown in Fig. 3. Relative expression of B2 gene had 2.8 folds compared to the non-salt stressed control followed by shmt and oee1 with 1.1 fold. (Fig 3a). Therefore B2, shmt and oee1 genes were selected for further analysis with more salinity levels

(0, 50, 100, 150, 200, 250 mM NaCl). B2 gene expression fold difference was 2.8 at 50 mM and raised up to its maximum value 6.2 at 100 mM NaCl then the expression decreased to 4.9 at 150 mM NaCl followed by 2.7 and 2.5 at 200 and 250 mM NaCl, respectively (Fig. 3b). Relative fold gene expression of shmt as follow 1.3, 4, 2.6, 1.4 and 1.6 and oee1 as follow 0.9, 2.4, 2.1, 0.9 and 0.6 at 50, 100, 150, 200 and 250 mM NaCl, respectively (Fig. 3c,d). It was observed that the expression pattern for all tested genes reached their maximum at 100 mM NaCl level, then declined till 250 mM. B2 and shmt had the highest fold increase upon salinity treatment, therefore were chosen for further analysis with cultivated barley.

Shahid *et al.*, (2012) screened salt-stress responsive genes from cotton leaves using the differential display technique. Gene expression was compared under control and salt stressed conditions by using a 107 primer combination. Total of 25 gene fragments were found to be up- regulated in response to salt stress. Out of 25 induced fragments, 12 were rejected as false positives after reamplification and quality control assay. The remaining 13 fragments were selected for cloning and sequence analysis using GenBank databases. Only 5 fragments ranging from 300 to 600 bp have significant homology to well-known proteins (*i.e.* protein kinase, proton gradient regulation, yeast cadmium factor, proteinase inhibitors, and expressed protein). Real time PCR studies confirmed over-expression of the identified transcripts in salt stressed samples as compared to control. Expression range for the 5 identified genes was from 1.5 to 5 folds.

Mohamed *et al.*, (2014) conducted differential display for Hibiscus sabdariffa under salt stress. A total of 81 primer combinations were used in the analysis. Nine up-regulated fragments were extracted, reamplified, cloned and sequenced. A homology search revealed that four transcripts showed significant homology with known genes. B1 and B2 fragments had homology with F-box protein in Arabidopsis, while B3 is similar to a putative serine/threonine protein kinase. Furthermore, B4 transcript had homology with a putative retrotransposon Ty3-gypsy protein. These four genes were validated using Real-time PCR analysis. B1 was down-regulated but not significantly differ than the control. B2, B3 and B4 were up-regulated from 2-2.5 folds under the stress.

Liu *et al.*, (2020b) employed transcriptome differential expression analysis of Prunellae Spica under salt stress. RNA-seq analysis was performed for Prunellae Spica treated with NaCl at different concentrations (0, 50, 150 and 200 mM). A total of 3857 genes were differentially expressed under low, medium and high salt stress, including 2456 up- regulated and 1401 down-regulated differentially expressed genes (DEGs). To test the quality of the differential expression analysis, 10 DEGs associated with salt stress response processes were randomly selected for qRT-PCR verification, and the results were compared with those of the RNA-seq. Out of the 10 DEGs, 3 DEGs had the maximum expression at 150 mMNaCl then the expression declined at 200 mMNaCl. This pattern of expression was similar to our study. On the other hand, 2 DEGs expression gradually increased with increasing salt concentration. The rest of the DEGs were inhibited more severely under medium NaCl treatment than under low or high NaCl treatment.

Conclusion

Chlorophyll fluorescence analysis was a valuble pre-screening analysis for wild barley seedlings before further molecular studies. Our molecular results clearly indicate the presence of potentialy useful salinity-tolerance genes that could be used further to improve cultivated barley counterpart. Out of the 7 identified genes, B2 gene had the highest level of increase in response to salinity, therefore could be a valuble target in further studies.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding Statement

The authors declare that the research is funded through the German-Egyptian research fund, Grant no. 5034, Egyptian Academy of Science, Egypt.

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