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Determining WRKY transcription factors related to salinity stress response in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.)

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ABSTRACT

The salinity is one of most common stress factors with devastating effects for wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L). The aim of this study was to determine the salt stress response associated with WRKY transcription factors by gene expression analysis. Seeds of *T. aestivum* L. cvs. Esperia and Bezozya, and *H. vulgare* L. cvs. Lord and Ramata were subjected to salt stress for 10 days with two concentrations of NaCl (1.0 % and 2.0 %). No germination was observed in 2.0 % NaCl treated seeds. Nearly all the *WRKY* genes studied were upregulated and downregulated in response to 1.0 % NaCl stress in relatively resistant and sensitive cultivars in comparison to control sets, respectively. Among the screened genes, the expression of *TaWRKY7*, *40*, *41*, *53*, *68*, *72* and *79* genes were increased in relatively resistant Esperia cultivar in wheat. Similarly, 7 of *WRKY* genes (*HvWRKY6*, *9*, *24*, *25*, *33*, *34*, *42*) were upregulated in relatively resistant Lord cultivar in barley. In terms of *WRKY* gene expression profile, since *TaWRKY7* and *TaWRK72* in wheat and *HvWRKY33* in barley increased significantly, these genes can be used as marker genes for further investigation of abiotic stress response. This study is a preliminary study in terms of providing an association between *WRKY* genes and salinity stress response of wheat and barley breeding cultivars in Turkey.

Anahtar Sözcükler:
Gene expression
WRKY
Salt stress
Hordeum vulgare L.
Triticum aestivum L.

Buğday (*Triticum aestivum* L.) ve arpada (*Hordeum vulgare* L.) tuzluluk stres yanıtı ile ilişkili WRKY transkripsiyon faktörlerinin belirlenmesi

ÖZET

Tuzluluk, buğday (*Triticum aestivum* L.) ve arpa (*Hordeum vulgare* L.) için yıkıcı etkileri olan en yaygın stres faktörlerinden biridir. Bu çalışmada, WRKY transkripsiyon faktörleriyle ilişkili tuz stresi yanıtının gen ekspresyon analizi ile belirlenmesi amaçlandı. *T. aestivum* L. cvs. Esperia ve Bezozya ile *H. vulgare* L. cvs. Lord ve Ramata tohumlarına iki NaCl konsantrasyonu (% 1.0 ve % 2.0) ile 10 gün boyunca tuz stresi uygulandı. % 2.0 NaCl uygulanan tohumlarda çimlenme gözlenmedi. Çalışılan hemen tüm *WRKY* genleri, kontrol gruplarına kıyasla % 1.0 NaCl stresine yanıt olarak nispeten dirençli ve hassas kültivarlarda sırasıyla yukarı ve aşağı yönde düzenlendi. İncelenen genler arasında, buğdayda nispeten dirençli Esperia kültivarında *TaWRKY7*, *40*, *41*, *53*, *68*, *72* ve *79* genlerinin ekspresyonu indüklendi. Benzer şekilde, arpada nispeten dirençli Lord'da 7 *WRKY* geni (*HvWRKY*6, *9*, *24*, *25*, *33*, *34*, *42*) indüklendi. *WRKY* gen ekspresyon profili açısından, buğdayda *TaWRKY7* ve *TaWRK72* ile arpada *HvWRKY33* genlerinde anlamlı derecede artış saptanması nedeniyle bu genler abiyotik stres yanıtının daha fazla araştırılması için marker genler olarak kullanılabilir. Bu çalışma, Türkiye'de ekimi yapılan buğday ve arpa çeşitlerinde *WRKY* genleri ve tuz stresi yanıtı arasında bir ilişki sağlaması açısından öncül bir çalışma niteliğindedir.

Keywords:
Gen ekspresyonu
WRKY
Tuz stresi
Hordeum vulgare L.
Triticum aestivum L.

1. Introduction

Cereals have great importance in biotechnology, medicine, industry and providing the food sources. The plantation of cereals takes an important place in the economy of many countries. According to food and agriculture organization (FAO) statistics, in Turkey, the production of wheat and barley were 20.600.000 and 6.700.000 tons, respectively, in the year 2016. Total yield production of wheat and barley worldwide were 749.460.077 and 141.277.993 tons, respectively. These data demonstrate that preservation, sustainability and improvement in wheat and barley resources are essential and crucial for the continuity of economic development.

Wheat is genetically closely related plant species of same taxonomic tribe, Triticale hexaploide Lart (Pourkheirandish and Komatsuda, 2007). Both crops are originated from primary agricultural production, including some parts of Turkey (Harlan and Zohary, 1966). Wheat has hexaploid genome (6n=52) of 17 Gb. Barley has diploid genome (2n=14) with a size of 5.1 Gb. Barley genome contains highly repetitive sequences 84 %) (Dawson et al., 2015). Barley is one of the oldest domesticated crops over 10 thousand years ago, and the origin of barley is thought in the Fertile Crescent area. All regions of Turkey are compatible with the cultivation of barley in terms of climate conditions. Barley is the world's fourth most important cereal after wheat, maize and rice. The species has great economic importance around the world and has been concerted into many biotechnological processes (Forster et al., 2000; Mayer et al., 2012).

Wheat and barley are exposed to biotic and abiotic stress factors such as heat stress, insects, drought and salinity. The drought and salt stress are one of the most serious abiotic stress factors in economically important cereals in global. Unfortunately, increased population and pollution, low land area for agriculture and global warming have increased the potential risk of drought and salinity stress, accordingly decreasing the output and quality of agriculture (Yadav et al., 2011; Hu and Schmidhalter, 2005). Nevertheless, plants have different kinds of mechanisms to improve resistance against stress factors. Tolerance responses are primarily managed by some important genes, gene families or metabolites including transcription factors (such as *DREB* and *WRKY*), microRNAs, hormones, co-factors and ions (Aktaş and Güven, 2005; Budak et al., 2015; Imadi et al., 2015).

WRKY transcription factors are related to gene families which have the possibility of playing a role in activating the signaling pathways and defense systems. This gene family is characterized by conserved 60 amino acids region with including the ‘’WRKY’’ amino acids at least one time. The transcription factor has zinc finger motif DNA binding site and up to a hundred *WRKY* genes have been characterized only for wheat and barley on databases (Rushton et al., 1996; Seki et al., 2002). However, there is limited data of definite association and annotation of *WRKY* transcription factors by type of abiotic and or biotic stress. *WRKY1*, *WRKY2*, *WRKY3*, *WRKY45* and *WRKY38* are among these transcription factors which have been related to some biotic stress factors in economically important crops (Rushton et al., 1996; Marè et al., 2004; Rushton et al., 2010).

The limited number of nucleotide sequence data described about the *WRKY* genes with specific characteristics on databases including National Center for Biotechnology Information (NCBI) and European Molecular Biology Laboratory (EMBL) limits plant biotechnological investigations. Likewise, there are no precise knowledge of *WRKY* genes for another plant species. For this reason, genes belong to the WRKY domain should be necessarily cloned and annotated for each plant species individually. As far as is known, wheat and barley cultivars used in this study have not been studied in terms of salinity stress responses, and totally 18 *WRKY* genes have not been previously associated with salinity stress in wheat and barley. In this study, it was aimed to reveal that *WRKY* genes are potential markers related to salinity stress in wheat and barley.

2. Material and Methods

2.1 Plant materials and salinity stress treatments

Seeds of eight barley cultivars (Aydan Hanım, Epona, Lord, Manava, Premium, Ramata, Tarm 92, Tokak157/37) and eight wheat cultivars (Alka, Antille, Bezozya, Canik 2003, Esperia, Forblanc, Midas, Quality) were used in this study. Wheat and barley seeds were disinfected with a 0.64 % sodium hypochlorite and 10 % ethanol for 5 min and washed three times with sterile deionized water. Then, 7 seeds were placed in 9 cm Petri dishes containing filter paper soaked with 3.0 ml of different NaCl concentrations (0 %, 1.0 % and 2.0 %), and germinated for 10 days at 25 °C for 16/8 h of light/dark photoperiod and 40 % relative humidity. Then, germination level of each individual cultivar was scored for both experiment and control sets on 0-9 scale (Badridze et al., 2009) (Table 1). 7 seeds were used in each experiment with three replicates. Two cultivars of each species were selected for further transcriptome analysis based on relatively resistant and sensitive characteristics.

2.2 Total RNA extraction and cDNA synthesis

Two relatively salinity resistant and two relatively salinity sensitive wheat and barley cultivars were used to reveal the potential regulation of totally 18 *WRKY* genes in response to salinity stress. For this purpose,

Çizelge 1. Tuzluluk çimlenme puanı ölçeği.
Table 1.Salinity germination score scale.

Score	Score Explanation
0	No germination
1	One root elongated or poor root development
2	Two roots elongated or more roots with brown tips
3	Three or more roots elongated, normal root development
4	Shoot less than 10 mm with green colour
5	Shoot elongated between 10 and 25 mm
6	First leaf protruded the coleoptile up to 1 cm
7	First leaf developed up to 3 cm from the coleoptile
8	First leaf developed up to 6 cm from the coleoptile
9	First leaf is longer than 6 cm from the coleoptile

total RNA was extracted using TriPure reagent (Roche, Switzerland). 100 mg of fresh leaves from 10-day-old of control and 1.0 % NaCl treated plantlets were homogenized via liquid nitrogen by using sterile mortar and pestle. 0.5 ml TriPure reagent was used to complete the homogenization step. After the homogenization, the manufacturer's recommendations were followed. The qualitative and quantitative analysis of total RNAs were carried out via 0.8 % agarose gel electrophoresis and spectrophotometer (Thermo, U.S.A.). cDNA synthesis was carried out by using commercial kit (Takara, Japan). 2 µg RNA was used as a starting amount. cDNA synthesis was carried out in a volume of 10 µl, including Oligo dT of 2.5 µM, random primers of 2.5 µM, 1X reaction buffer, reverse transcriptase enzyme of 10 U. ¼ diluted cDNAs were used in gene expression analysis.

2.3 Quantitative real time polymerase chain reaction (qRT-PCR) assays

qRT-PCR primers of 10 *WRKY* genes for wheat and 8 *WRKY* genes for barley are listed in Table 2. *α-actin* was used as endogenous control. Relative quantification method was used in salinity assays. QuantStudio 5.0 (Applied Biosystem, U.S.A.) system was used in qRT-PCR assays. Sybr Green I was used as fluorophore. qRT-PCR experiments were done in microtubes with 10 µl reaction volume containing 1X Sybr Green I mix (Takara, Japan), 2 pmol primers and 2 µl cDNA corresponding to 50 ng total RNA. Cycling conditions

Çizelge 2. Çalışmada kullanılan primerler.
Table 2. Primers used in this study.

Gene	NCBI Accession	Forward sequence (5'–3')	Reverse sequence (5'–3')	Amplicon size (bp)
TaActin	KC775780	GGCACACTGGTGTCATGGT	GCGCCTCATCACCAACATA	124
TaWRKY4	EU665433	CTGAGCTACAGCGGGTGAG	GTGACCATGTCGGTGAACTG	104
TaWRKY7	EU665436	AGCTCTCCATTGCCTTCTTC	ACCATTTCTTGGTTCGTTGG	112
TaWRKY27	EU665431	GCTCCTCACCTCCAGTATCC	GGAGTAGGGCTGCCTCTG	133
TaWRKY33	EU669663	CGGCAATAACAGCCACTACA	GTTGCTCTCCTCGCTCTTG	94
TaWRKY40	EU665455	CACCTTTCAGCAGGATGAGC	AGTTTGCTTGAGCGTTGACC	101
TaWRKY41	KF195931	CAGCACGGATTTCTTCAAAA	CCATCATCGTGACCCTCAAT	101
TaWRKY53	KC174859	GCCTCTTTGGCTTCTCCTTT	CTGCTGCTGATGTTCCTTGA	95
TaWRKY68	EF397617	CTCCTCGTCTCCTCCCTCTC	GAGATCACCTTGCGGAACTT	116
TaWRKY72	KT373801	AGCCCTCCAACTCAAGGAT	CTCCCTTTTCTCGCCTTTCT	110
TaWRKY79	JX047374	TGGACGAGCAGTGGATGA	CGTGGTTCTTCTTGGAAGACAT	138
HvActin	AY145451	GGCACACTGGTGTCATGGT	GCGCCTCATCACCAACATA	90
HvWRKY6	EF488106	CGAAGGTCATTGTGCTGTTG	CTGTACCCATCGCTCATCTT	101
HvWRKY9	DQ840408	AGGTTTCAGCTCATGCACCA	TGACACCCTTGCCACCACTA	106
HvWRKY24	DQ863108	CATGAGCAGAGCACCATCT	GACATCATCCGCACCTGTAT	110
HvWRKY25	DQ863109	CATCATGGAGGTCCAAGCAA	ACCCGACAATGTCCTTCTGG	114
HvWRKY33	DQ863117	CTGCAACTTTCCCAGGTACT	GGGTCGCTGTGATCTTTCT	96
HvWRKY34	DQ863118	AACCAACAGAGCGACATAGG	CTGTCGGTCTCCATCTTGAC	98
HvWRKY42	DQ863125	AGTGAAGGACAGTGCTGATG	GGTCTTCCTCGTTCTCTTCC	104
HvWRKY46	AY323206	ATTCGCCTGGTATGGTTGAG	TCCTCCTCCTCAGTAGCATC	106

were as pre-denaturation at 95 ºC for 2 min, 45 cycles of 95 ºC for 10 sec, 58 ºC for 15 sec, 72 ºC for 20 sec. Melting curve analysis was performed at the end of cycling. Standard series were conducted on 5 logarithmic phases. $2^{-\Delta\Delta CT}$ normalization formula developed by Livak and Schmittgen (2001) was used to analyze fold changes in gene expression. qRT-PCR assays were replicated thrice.

2.4 Statistical analysis

The statistical output related to qRT-PCR results were analyzed by GraphPad Prism 5.0 (Dr. Harvey Motulsky, U.S.A.) software using One-way analysis of variance (ANOVA) followed by Tukey's post hoc test. One-way ANOVA with least significance difference (LSD) test function at $P \leq 0.05$ in R 3.5.2 statistical software with RStudio (Version 1.1.463) and the package agricolae was applied for germination score analysis. Two-way ANOVA analysis was also carried out to check the efficiency of investigations.

3. Results and Discussion

Total of eight cultivars for each species were germinated for 10 days at 25 ºC and 16/8 h of light/dark photoperiod. Salinity treatment was applied by 0 %, 1.0 % and 2.0 % NaCl concentrations. According to the findings, no germination was observed in 2.0 % NaCl treated experiment sets. Significantly decreased to 2.0 % NaCl treatment in wheat and barley cultivars was an expected result since NaCl causes strong salt stress for many plant species (Badridze et al., 2009; Almodares et al., 2014; Jain et al., 2016; Yörük et al., 2018). Thus, further transcript analysis was carried with 0 % and 1.0 % NaCl treated sets. Based on the germination scores, *H. vulgare* L. cv. Ramata and *T. aestivum* L. cv. Bezozya were selected as relatively resistant while *H. vulgare* L. cv. Lord and *T. aestivum* L. cv. Esperia were selected as relatively sensitive cultivars (Table 3).

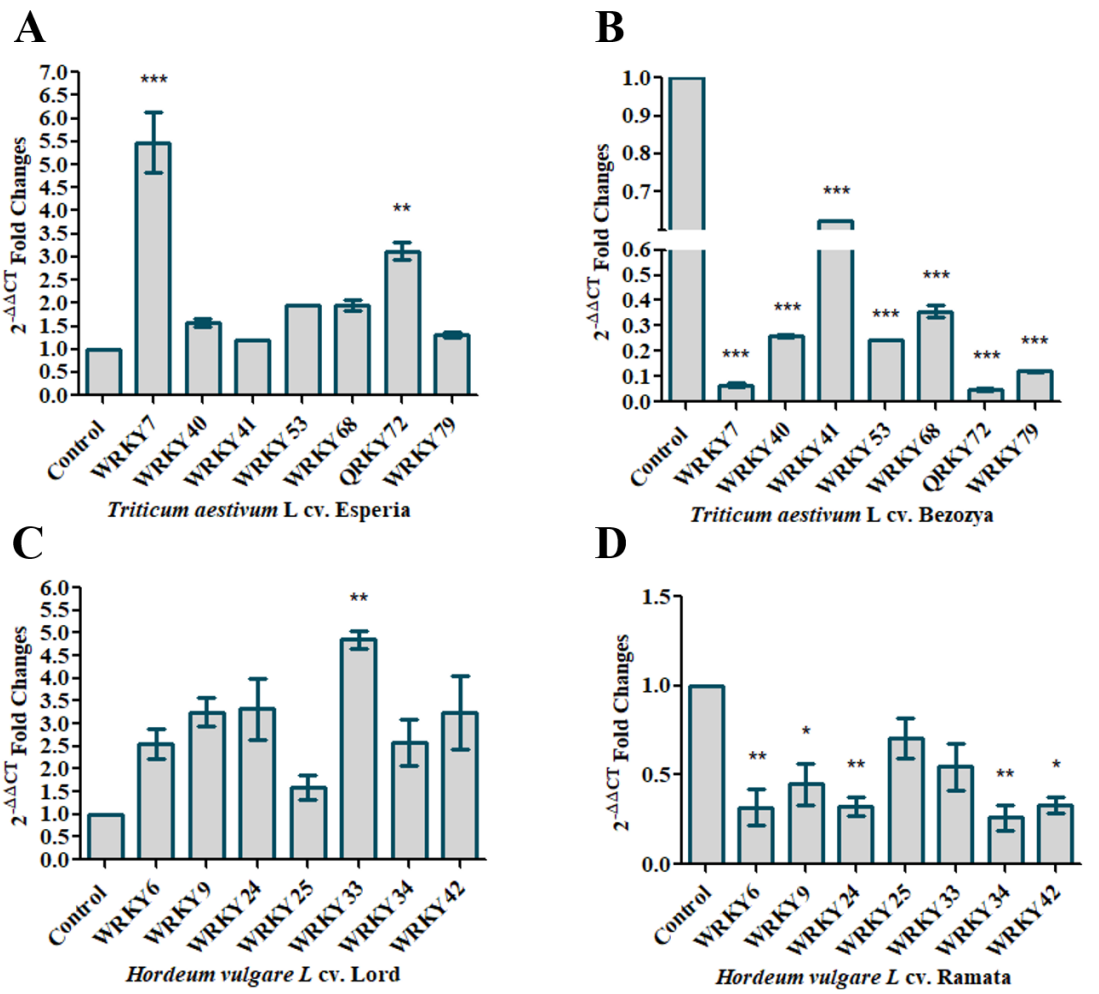
In qRT-PCR results of wheat, 7 of 10 genes (*TaWRKY7*, *TaWRKY40*, *TaWRKY41*, *TaWRKY53*, *TaWRKY68*, *TaWRKY72* and *TaWRKY79*) were increased compared with control in Esperia (relatively salinity resistant) and down-regulated in Bezozya (relatively salinity sensitive) (Figure 1 A, B). Two-way-ANOVA analysis showed that each gene revealed significant differences ($P<0.001$). Remaining three genes showed no significant changes in response to salinity treatment. The fold changes in gene expression ranged from 1.19±0.01 (*TaWRKY41*) to 5.47±0.66 (*TaWRKY7*) in Esperia. Down-regulation values ranged from 0.048±0.004 (*TaWRKY72*) to 0.623±0.001 (*TaWRKY41*) in Bezozya (Figure 1 A, B). Results from this study showed that among related *WRKY* genes, 7 genes can be used in further salinity responses related investigations in wheat. Especially, *TaWRKY7* and *TaWRKY72* genes can be used in more detailed and comprehensive studies such as genetic transformation and marker assisted selection of wheat and genetically closely related plant species. *HvWRKY6*, *HvWRKY9*, *HvWRKY24*, *HvWRKY25*, *HvWRKY33*, *HvWRKY34* and *HvWRKY42* genes were upregulated in experiment sets in barley. The fold changes in gene expressions were ranged from 1.602±0.38 (*HvWRKY25*) to 4.85±0.28 (*HvWRKY33*) in Lord. Down-regulation values were ranged from 0.26±0.1 (*HvWRKY34*) to 0.707±0.161 (*HvWRKY25*) in Ramata (Figure 1 C, D). As well as in wheat qRT-PCR analysis, 7 genes were up-regulated and down-regulated in salt resistant and sensitive cultivars, respectively. Particularly, *HvWRKY33* with 4.85±0.28 fold increase for barley could be used as marker genes in further abiotic stress response investigations. In a previous study we reported transcript profiles of 8 *WRKY*s upon *Fusarium culmorum* infection in two barley cultivars. Similarly, we found that the transcript level of *HvWRKY33* was significantly upregulated in both cultivars (Uluhan et al., 2019). Totally 14 genes could stimulate the expression of abiotic stress response related genes in wheat and barley. This study is important in terms of providing an association between *WRKY* genes and salinity stress response in wheat and barley. Also, it's the first report to show these 14 *WRKY* genes' fold increase in salinity treated plants.

The results obtained from current study reveal that totally 14 *WRKY* genes could be used in further plant molecular biology researches. Particularly, three *WRKY* genes showing high level of mRNA abundance alterations in response to salinity stress may play a key role in activating the genes which are related to salinity stress responses in cereals. Several previous studies have showed that qRT-PCR analysis presented reliable and reproducible data associated with alterations in gene expression (including transcription factor coding genes) in response to salinity stress in economically important crops (Robatzek and Somssich, 2002; Bagdi et al., 2015). In this study, 14 *WRKY* transcription factors coding genes have been associated to salinity stress response in relatively salt stress sensitive and resistant wheat and barley cultivars. In contrast to previous studies which represent *WRKY* genes association with biotic stress responses (Eulgem et al., 2000; Robatzek and Somssich, 2002; Rushton et al., 2010; Niu et al., 2012; Wang et al., 2015), this study shows that WRKY transcription factors may play important role in abiotic stress response in major cereal species. The findings are important as this is the first report to show these 14 *WRKY* genes' fold increase in salinity treated plants. The findings are also important in determining the salinity stress sensitivity of barley and wheat cultivars which are not tested for their abiotic stress response capacity previously.

Çizelge 3. % 1.0 NaCl uygulamasının farklı arpa ve buğday çeşitlerinde çimlenme özellikleri üzerine etkisi.
Table 3. Effect of 1.0 % NaCl treatment on germination attributes in different barley and wheat cultivars.

Barley Cultivars	1.0 % NaCl	Wheat Cultivars	1.0 % NaCl
Lord	4.72±0.23 a	Esperia	3.00±0.26 a
Tarm 92	4.27±0.35 ab	Canik 2003	2.36±0.24 ab
Premium	3.45±0.28 bc	Alka	2.27±0.23 ab
Aydan Hanım	4.81±1.16 a	Midas	1.81±0.26 bc
Ramata	1.54±0.41 d	Bezozya	0.27±0.14 d
Tokak 157/37	1.81±0.35 d	Antille	0.45±0.15 d
Manava	2.54±0.36 cd	Quality	0.63±0.20 d
Epona	3.27±0.35 bc	Forblanc	1.18±0.12 cd

The data presented are the means ± standard errors (SE) of three replicates. Values in vertical columns followed by different letters are significantly different at $P \leq 0.05$.



Şekil 1. *T. aestivum L. cv. Esperia (A), T. aestivum L. cv. Bezozya (B), H. vulgare L. cv. Lord (C) ve H. vulgare L. cv. Ramata (D)'da WRKY gen ekspresyonuna ilişkin kat değişimleri (*P < 0.05, **P < 0.01, ***P < 0.001).*

*Figure 1. Fold changes in WRKY gene expression related to T. aestivum L. cv. Esperia (A), T. aestivum L. cv. Bezozya (B), H. vulgare L. cv. Lord (C), and H. vulgare L. cv. Ramata (D) (*P < 0.05, **P < 0.01, ***P < 0.001).*

4. Conclusions

Wheat and barley are economically important crops worldwide and they are subjected to salinity stress in many agro-ecological regions. The identification of the marker genes related to salinity response and/or determining the salinity stress resistant cultivars have great importance in agricultural practices worldwide. For these purposes, several traditional and modern techniques have been still used in fields and *in vitro* investigations. In this study, qRT-PCR based gene expression analysis revealed particularly 3 important marker genes related to salinity stress response in the wheat and barley cultivars. The stress response capacities of wheat and barley cultivars from any regions worldwide can be detected by using particularly *TaWRKY7*, *TaWRKY72* and *HvWRKY33* genes. Additionally, further studies could include more cultivars and undefined genes which can be related to abiotic stress response in order to obtained more detailed and comprehensive data which can be useful in plant molecular biology research area.

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