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## Revision of the barley *WRKY* gene family phylogeny and expression analysis of the candidate genes in response to drought

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### Abstract

The WRKY belongs to an important plant specific transcription factor families which are involved in response to various environmental stresses, as well as in growth and developmental processes. In the present report, a genome-wide identification and characterization of *WRKY* gene family in barley led to revision of HvWRKYs to 93 members. The phylogenetic tree was also reconstructed based on the full-length WRKY protein sequences in barley and *Arabidopsis*. HvWRKYs were classified into three major groups (I, II, and III) and group II was further divided to 5 subgroups (a to e). HvWRKYs were named after this classification. Interestingly, some specific motifs were discovered in subgroups IIa, IIb, and III. Analyzing the available microarray data revealed eight candidate *WRKY* genes which were up-regulated under drought and salinity stresses compared to the optimum conditions at seedling stage in barley. Expression profiles of these *WRKY* genes were validated by quantitative real-time PCR. All the investigated candidate genes (*HvWRKY\_IIC2*, *HvWRKY\_III11*, *HvWRKY\_IIB2*, *HvWRKY\_IID4*, *HvWRKY\_III23*, *HvWRKY\_IIA5* and *HvWRKY\_IIC19*) except for *HvWRKY\_I8* were significantly up-regulated by drought stress at the seedling stage in drought-tolerant genotype, indicating their role in drought tolerance. We hope the presented information would be helpful toward achieving drought tolerant cultivars through genetic engineering or molecular breeding.

*Additional key words:* *Arabidopsis thaliana*, chromosomal location, drought tolerance, *Hordeum vulgare*.

### Introduction

Putative transcription factor (*TF*) genes constitute 7 % of all plant genes. TFs are proteins which play a major role in gene expression regulation (Cowell *et al.* 1992). When these specific proteins bind to the upstream of transcription start site (gene regulatory regions), the transcription activity will be affected (Ryu *et al.* 2007). The WRKY family is an outstanding higher plant TF type, which has not been found in both fungal and animal genomes (Zhang and Wang 2005, Rushton *et al.* 2010). Based on the phylogenetic tree classification, *Magnoliophyta* have more WRKY members compared to *Pinophyta*, *Pteropsida*,

and *Bryophyta*. These TFs play a very important role in the regulation of *Magnoliophyta* gene expression (Berri *et al.* 2009). Phylogenetic analysis of WRKY family in *Arabidopsis thaliana* and *Oryza sativa* genomes has modified initial classification (Wu *et al.* 2005, Xie *et al.* 2005, Zhang and Wang 2005).

Based on the number of WRKY domain (WD) sequences (tryptophan-arginine-lysine-tyrosine) and the identity of the zinc-finger motif, the WRKY genes are classified into three main groups (I, II, and III). Furthermore, group II was divided into five subgroups (IIa, IIb, IIc, IId, and IIe). All WRKY proteins have one (groups II and III) or two (group I) conserved WDs which have a signature peptide

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*Abbreviations:* ABA - abscisic acid; TF - Transcription factor; WD - WRKY domain (sequence tryptophan-arginine-lysine-tyrosine).

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sequence of WRKYGQK at the N-terminus. Also, a C<sub>2</sub>H<sub>2</sub>- (groups I and II) or a C<sub>2</sub>HC-type zinc-finger motif (group III) at the C-terminus, with a total length of approximately 60 amino acids (Eulgem *et al.* 2000). Variants of the WRKYGQK signature motif comprise WRKYGEK and WRKYGKK, with several atypical WRKY motifs suggesting the consensus W(R/K)(K/R)Y (Xie *et al.* 2005). Most WRKY proteins bind preferentially to the W-box DNA sequence -(C/T)TGAC(T/C)- (Rushton *et al.* 1995, De Pater *et al.* 1996, Wang *et al.* 1998, Eulgem *et al.* 1999). Nevertheless, some WD sequences can present different binding preferences such as binding with either the SURE (sugar-responsive *cis*-element) or the W-box (Sun *et al.* 2003, Van Verk *et al.* 2008). Invariable WRKY amino acids signature as well as cysteine and histidine residues of the WD, which tetrahedral coordinate a zinc atom, are necessary for W-box dependent binding activity (Maeo *et al.* 2001).

While WRKY TFs have been implicated in some developmental processes (Johnson *et al.* 2002, Lagac and Matton 2004, Miao *et al.* 2004), their key functions are defense responses against biotic and abiotic stresses (Johnson *et al.* 2002, Zhou *et al.* 2008, Jiang and Deyholos 2009, Pandey and Somssich 2009, Wu *et al.* 2009, Prabu *et al.* 2011). Most of the genes in group IIa and IIc are involved in biotic and abiotic responses. Genes in group I play a role in developmental processes and biotic and abiotic stress responses in crops, while the functions of most genes in group III should be characterized (Chen *et al.* 2017). In addition, all WRKY subfamilies have drought and salt stress responsive members. Also, genes in group IIc might be the crucial TFs involved in drought and salt stress responses in crops (Chen *et al.* 2017). Some WRKY genes in several plant species are highly expressed during seed development stage (Bakshi and Oelmüller 2014). Previous studies have suggested that WRKY TFs have major roles in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), which are important innate immune systems in the plant (Pandey and Somssich 2009). In response to stress, WRKY TFs are involved in ABA mediated signaling pathway (Jiang *et al.* 2017). Members of WRKY TFs family have been identified in some plants including 92 *TaWRKY* in *Triticum aestivum* (Zhu *et al.* 2013), 99 *OsWRKY* in *Oryza sativa* (Ross *et al.* 2007), and 72 *AtWRKY* in *Arabidopsis thaliana* (Wu *et al.* 2005).

Barley (*Hordeum vulgare* L.) is a major food source with harvestable yields in harsh environments and one of the main cereal crops in developing countries. It has the highest drought stress tolerance among other crops including rice, maize and wheat, and can considerably adapt to various environmental conditions (Ullrich 2011). The barley genome (Morex genotype) has been completely sequenced (Consortium 2012), and this fact provides an excellent opportunity to raise research on barley. In the present study, we attempted to recognize members of WRKY TFs family, and their characteristics in barley *via in silico* approaches. We also conducted gene expression analysis of their candidate genes involved in drought stress tolerance in barley.

## Materials and methods

### Identification of WRKY gene family members in barley:

Barley (126) and *Arabidopsis* (72) TF sequences were downloaded from the plant TFs database (*PlantTFDB v. 4.0*; <http://planttfdb.cbi.edu.cn/>; Jin *et al.* 2017). WRKY proteins from each *Arabidopsis* subfamily were used as a query for *tBLASTn* analysis in the international barley sequencing consortium (IBSC) IPK BLAST server (<http://webblast.ipk-gatersleben.de/barley/>) (Consortium 2012) against full length cDNA, high confidence (HC) and low confidence (LC) databases. Further, all sequences with a unigene accession number (60 unigene) for barley WRKY proteins were downloaded from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>), as well. Additionally, hidden Markov model (HMM) profiles of WD (PF03106) were obtained from Pfam database (<http://pfam.xfam.org/>; Finn *et al.* 2016) and used to find putative WRKY proteins against barley HighConf\_genes\_MIPS\_23Mar12\_ProteinSeq and barley LowConf\_genes\_MIPS\_23Mar12\_ProteinSeq (Consortium 2012) using HMMER v. 3.1b1 where the e-value threshold was set at 1. Finally, redundant sequences were removed manually. To find all the *HvWRKY* transcript sequences, cDNA and protein sequences were used as queries for *BLASTn* and *BLASTp* searches against *Ensembl* plants database (IBSC v. 2; <http://plants.ensembl.org/index.html>), respectively. All non-redundant sequences were checked in the Pfam database for existence of WD.

### Chromosomal Location of HvWRKYs on barley genome:

The cDNA and protein sequences were employed as the queries for *BLASTn* and *tBLASTn* searches against *EnsemblPlants* database (<http://plants.ensembl.org/index.html>) to find the location of *HvWRKY* genes on barley chromosomes, respectively. The distribution of *HvWRKYs* was created using the *MapChart v. 2.3* software (Voorrips 1994).

### Phylogenetic analysis:

Multiple sequence alignment (MSA) of the WRKY transcription factors for full-length protein sequences from *Arabidopsis* and barley were obtained using *ClustalX* program (Larkin *et al.* 2007). Phylogenetic tree was constructed using the neighbor-joining (NJ) method by *MEGA7* (<http://www.megasoftware.net/>; Kumar *et al.* 2006), and 1 000 bootstrapping replicates. The Gonnet matrix method was used to compute the evolutionary distances and estimate the number of amino acid substitutions per site.

### Detection of additional conserved motifs of WRKY proteins:

Recognition of the *HvWRKY* protein motifs was performed using motif elicitation tool (*MEME*; v. 4.11.4; <http://meme-suite.org/tools/meme>; Bailey *et al.* 2009). The setting options included any number of repetitions, ten and sixty amino acids for the minimum and maximum width of a motif, respectively, and 15 for the maximum number of motifs to find. All discovered motifs by *MEME* were scanned in *InterPro* database (<http://www.ebi.ac.uk/interpro/>; Bailey *et al.* 2009).

**In silico WRKY gene expression survey:** Microarray data, 22 K Affymetrix gene chip array (*Barley 1*), were downloaded from GEO (<http://www.ncbi.nlm.nih.gov/gds/>) containing accession numbers GSE3170, GSE6990, GSE15970, and GSE17669 for drought, plus GSE3097 and GSE5605 for salinity. Based on the annotation results, 22 probes were detected for *HvWRKYs* in the Affymetrix *barley 1* chip. Affymetrix raw data [cell intensity (CEL)] files were used as input files in Expression Console software (v. 1.3.1.187). The raw data was normalized using robust multi-array analysis (RMA) through sketch quantile normalization approach (Irizarry *et al.* 2003). Then, the Flexarray software package (v. 1.6.1) was employed to statistically analyze the normalized array data via analysis of variance (ANOVA). A hierarchical cluster map was created (Pearson method) to find the differentially expressed genes using online heat mapper (<http://www.heatmapper.ca/expression/>).

**Plants and drought stress treatments:** Seeds of spring barley (*Hordeum vulgare* L.) cv. Yousef (a drought-tolerant Iran native cultivar) and Morocco 9–75 (a drought-susceptible international cultivar) were obtained from the Seed and Plant Improvement Institute (SPII) and the International Center for Agricultural Research in the Dry Area (ICARDA), respectively (Ceccarelli 1994, Abedini *et al.* 2017). The plant growth and water treatment were done as previously described (Abedini *et al.* 2017). Briefly, the culture was performed in a greenhouse located at the Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran (a 12-h photoperiod with an average photosynthetic active radiation density of 180  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and day/night temperatures of 23/20 °C), as a complete random design (CRD) with three biological replications (a replication included a pot containing 6 plants). Two levels of water irrigation including 70 and 10 % of water holding capacity of soil were considered as optimum and severe drought stress conditions, respectively. The samples were obtained from young fully expanded leaves of both 36-day-old genotypes grown under optimum conditions or a gradual drought stress for 21 days. Then, leaves were rapidly dipped into liquid nitrogen and stored at -80 °C until RNA extraction.

**Expression study of *HvWRKYs* genes under drought stress:** Total RNAs were isolated using Trizol reagent (Invitrogen, Carlsbad, USA) and were treated with RNase-free DNase (Geneall, Seoul, Korea) according to the manufacturer's instruction. RNA samples were reverse transcribed to cDNA with oligo (dT)<sub>18</sub> and random primers using cDNA synthesis kit (Takara, Tokyo, Japan).

Gene-specific primers for eight *HvWRKY* genes (*HvWRKY Iic2*, *HvWRKY III11*, *HvWRKY Iib2*, *HvWRKY IId4*, *HvWRKY III23*, *HvWRKY I8*, *HvWRKY Ila5*, and *HvWRKY Iic19*) were designed using Oligo software (Rychlik 2010) that are described in Table 1 Suppl1. Real time qRT-PCR was performed on a LightCycler®96 (Roche, Mannheim, Germany) using SYBR Green Supermix (Takara). The reactions were performed using the following program: 95 °C for

5 min and 40 cycles (95 °C for 30 s, 58° or 61°C for 30 s and 72 °C for 30 s). Barley *Actin* gene was used as the internal control (Tommasini *et al.* 2008). Each reaction was carried out in three independent biological replicates with the relative expression was calculated using the 2<sup>- $\Delta\Delta\text{CT}$</sup>  method (Schmittgen and Livak 2008) by REST 2009 software (Pfaffl *et al.* 2009). Gene expression in drought-sensitive genotype under optimum condition was used as a calibrator for calculating the relative gene expression. The relative gene expressions of candidate genes were analyzed by a complete random design where the average values of treatments were calculated by the least significant difference (LSD) test using SAS statistical package ( $P < 0.01$ ) (Afifi *et al.* 2003).

## Results and discussion

In this study, 93 *WRKY* gene loci were discovered encoding 497 proteins in *Hordeum vulgare*. In the first study of *WRKY* gene family in barley, 46 members were recognized and named from *HvWRKY1* to *HvWRKY46* (Mangelsen *et al.* 2008). Then, fifteen novel *HvWRKY* members, *HvWRKY57* to *HvWRKY61*, were found (Meng and Wise 2012). A recent study in barley discovered 34 additional *WRKY* TFs from *HvWRKY62* to *HvWRKY95* (Liu *et al.* 2014). Notably, previous studies revealed that *HvWRKY1* was also published as *HvWRKY38* (Eckey *et al.* 2004, Mangelsen *et al.* 2008). Further, *HvWRKY56* was the full-length protein of *HvWRKY8* (Liu *et al.* 2014).

Our investigation revealed that *HvWRKY35* (DQ863119) and *HvWRKY41* (AK365469/DQ863124) reported in the previous studies belong to the same unigene accession (Hv.9019). According to the results, *HvWRKY72* (MLOC\_3853) includes the complete protein and coding sequence version of *HvWRKY49* (JQ806390) and, the genes are mapped to the same locus (HORVU3Hr1G081000) on the barley genome. Although, there are different unigene accessions for *HvWRKY3* (AK359706) and *HvWRKY52* (AK368514); (Hv.13567 and Hv.32682, respectively), the protein and coding sequence alignments indicated that they are different transcripts of HORVU5Hr1G065420. In addition, *HvWRKY31* (DQ863115/MLOC\_5971) and *HvWRKY95* (MLOC\_15430) were completely similar to different transcripts of HORVU1Hr1G069620. We also found three novel *WRKY* TFs. The additional information on *HvWRKY* TFs including previous and new *WRKY* names, chromosomal locations, unigenes, cDNA, and protein accession numbers, *Ensembl* and *MIPS* accession numbers, number of the introns, as well as the protein length and position of WD have been provided in Table 1 Suppl2.

The full length protein sequences of *WRKY* TFs in barley and *Arabidopsis* were aligned and the unrooted phylogenetic tree was constructed. Based on the number of *WRKY* domains, the sequence similarity of both *WRKY* domain and zinc-finger motif, as well as on the phylogenetic tree, *HvWRKYs* were classified into three main groups (I, II, III) which is in accordance to previous reports in barley

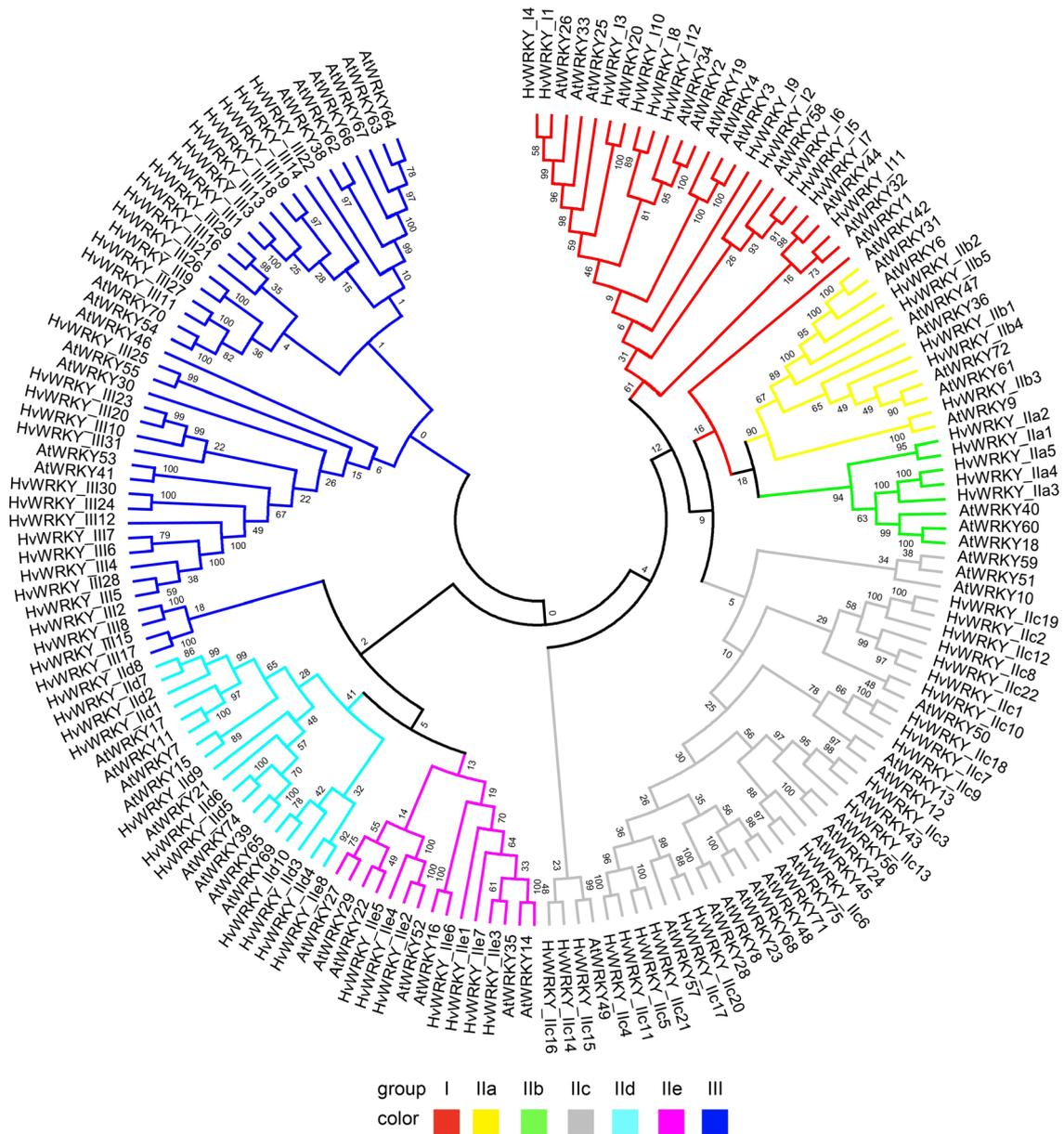


Fig. 1. An unrooted phylogenetic tree of WRKY proteins from barley (*HvWRKY*) and *Arabidopsis* (*AtWRKY*). Protein sequences were aligned using *ClustalX* and the unrooted phylogenetic tree was constructed using *MEGA7* by the neighbor-joining method with 1 000 bootstraps. The WRKY proteins were classified into 7 subgroups (I, IIa, IIb, IIc, IId, IIe, and III).

Chromosomal locations of 10 genes (*HvWRKY\_112*, *HvWRKY\_IIb5*, *HvWRKY\_IIc19*, *HvWRKY\_IIc20*, *HvWRKY\_IIc21*, *HvWRKY\_IIc22*, *HvWRKY\_III28*, *HvWRKY\_III29*, *HvWRKY\_III30*, and *HvWRKY\_III31*) were indistinct on the barley genome. However, the rest of *HvWRKY* genes (83 out of 93) were physically mapped on barley chromosomes according to the *Ensembl* database (Fig. 2). The *HvWRKY* members were distributed on all chromosomes of barley, whereas chromosome 3 contained the maximum number of *HvWRKY*s, having 21 of them (23 %), followed by 17 *HvWRKY*s (18 %) on chromosome 5, and 5 *HvWRKY*s (5 %) on chromosome 6.

and other plants (Eulgem *et al.* 2000, Mangelsen *et al.* 2008, Meng and Wise 2012, Liu *et al.* 2014). Furthermore, group II was divided into five (a, b, c, d, and e) subgroups, respectively. In barley, the zinc finger motif in group II was similar to group I, with the CX4-5CX22-23HXH pattern, while the zinc finger in group III had CX7CX23HXC pattern based on WRKY domain alignment (Fig. 1 Suppl.). *HvWRKY* genes were given a generic name based on their

classification (*HvWRKY\_I*, *HvWRKY\_IIa*, *HvWRKY\_IIb*, *HvWRKY\_IIc*, *HvWRKY\_IId*, *HvWRKY\_IIe*, *HvWRKY\_III*) with an additional number (e.g., 1, 2, 1...) and also decimal for different transcripts encoded by the same locus (e.g., *HvWRKY\_11.1* - *HvWRKY\_11.2*) (Table 1 Suppl2.).

Comparison between the unrooted phylogenetic tree in this study and the preceding report by Liu *et al.* (2014) revealed that all the members were placed in the same

subgroups except four members (*i.e.* HvWRKY\_IId3, HvWRKY\_IId4, HvWRKY\_III20, and HvWRKY\_III29). HvWRKY\_IId3 and HvWRKY\_IId4 belonged to the IId in the aforementioned report while they were clustered into IId subgroup in this study. These members joined AtWRKY65 which has classified in IId or IId subgroups according to two previous studies (Wu *et al.* 2005, He *et al.* 2012). Anyway, IId and IId subgroups always cluster into a single clade. In addition, HvWRKY\_III20 and HvWRKY\_III29 were located in subgroup III in the present study while they were classified in IId and IId in the former report, respectively (Liu *et al.* 2014). AtWRKY41, AtWRKY53, AtWRKY54, AtWRKY55, and AtWRKY70 belonging to the group III based on the phylogenetic tree of *Arabidopsis* (Wu *et al.* 2005) were joined to the HvWRKY\_III20 and HvWRKY\_III29.

Group I contained 12 and 13 WRKYs in barley and *Arabidopsis*, respectively (Fig. 1). AtWRKY1 joined to group II in *Arabidopsis* and barley uprooted phylogenetic tree but it has two WRKY domains thereby was classified into group I. All members in group I had two WRKY domains except for HvWRKY\_I10 with one WRKY domain. This phenomenon was observed in foxtail millet (Zhang *et al.* 2017). Also, 6 out of 31 WRKY members belonging to group I in radish had one WD (Karanja *et al.* 2017). This suggests that they might have either experienced domain loss or gain events during evolution (Ross *et al.* 2007). According to the studies on unicellular green alga *Chlamydomonas reinhardtii* with a great genetic similarity with plant ancestors, there is only one WRKY gene which is a part of group I (with two WDs). This can indicate that group I is the actual ancestor of WRKY family from which other groups of the family have been evolved (Goel *et al.* 2016). Searching in GeneBank revealed that there are WRKY genes which are similar to group I in two non- photosynthetic eukaryotic organisms: one in *Dictyostelium discoideum* (AAO52331) and the other one in a protozoan called *Giardia lamblia* (EAA40901). This generally confirms the hypothesis of group I as being the true ancestor of WRKY genes. WRKY genes originated about one and a half to two billion years from early eukaryotes, prior to their transfer into the plant kingdom. However, there is no logical explanation behind their expansion into plants kingdom while being absent in yeasts and animals (Ülker and Somssich 2004).

Group II was classified into five subgroups in barley: IIa, IIb, IIc, IId, and IId (Fig. 1) as in the classification of WRKY family in wheat, rice, and *Arabidopsis* (Wu *et al.* 2005, Rushton *et al.* 2010, Zhu *et al.* 2013). Subgroup IIa, IIb, IIc, IId, and IId contained 5, 5, 22, 10, and 8 members, respectively. Similar to *Jatropha curcas*, 10 out of 12 members in group II in barley had an extra plant zinc domain (pfam10533) upstream of the WRKY (Xiong *et al.* 2013). In other studies, the evolution of group II from group I have been acknowledged given the loss of amino terminal WD. The results also suggested that group II has been evolved from the carboxyl terminal WD of group I (Zhang and Wang 2005). In proteins of group I, carboxyl domain and amino domain help DNA-binding activities and specialization of the bind, respectively. So, because of

the transcription factor DNA-binding nature, the evolution of group II from carboxyl domain seems to be logical.

On average, about 20 % of WRKY gene family in higher plants belongs to group III while the members of this group do not exist in moss (*Physcomitrella patens*). This indicates that group III of WRKY genes has evolved following groups I and II (Dong *et al.* 2003, Kalde *et al.* 2003) and exists only in higher plants. Interestingly, HvWRKY\_III24 and HvWRKY\_III30 had two WDs, but they were classified in group III given the zinc finger motif pattern (C2-H2). 33 % (31 members) where 18 % WRKY members in barley and *Arabidopsis* belonged to group III, respectively (Fig. 1). Compared to *Arabidopsis*, group III in rice was far more active in terms of evolution since in the sequences of the group III of rice, additional duplication has been observed (Eulgem *et al.* 2000) with 34 % of WRKY genes falling into group III in rice (Ross *et al.* 2007). Hence, the diversity in the number of WRKY members in group III was represented by an expansion of WRKY members of this group in barley. Alignment of multiple HvWRKY domain amino acid sequences (Fig S1) showed group I and II had histidine (H) in the conserved domain whereas group III had cysteine (C). Not only, HvWRKYIII\_2, HvWRKYIII\_8, HvWRKYIII\_15, and HvWRKYIII\_17 followed group III conserved domain pattern, but also they shared same amino acid motifs pattern according to the Fig. 2 Suppl. Therefore, these genes were classified into group III.

The additional conserved motifs (15 putative motifs) were discovered by MEME program using the longest proteins transcript of all members of WRKY subfamily in barley. Typically, most WRKY members within the same cluster in phylogenetic tree shared the same motif compositions. Therefore, these WRKY proteins have similar functions (Xie *et al.* 2018). Predictably, most of the HvWRKYs in the same subgroups had similar motif arrangements (Fig. 2 Suppl.). Therefore, the reliability of the grouping in barley was supported by phylogenetic analysis results along with the similarities of conserved motif compositions in each subgroup. The motif 1 and motif 9 contained conserved WRKYGQK domain where at least a number of them were present in all barley WRKY amino acid sequences. The motif 7 consisted of plant zinc domain which only existed in most of the subgroup IId members. On the other hand, biological functions of other motifs were unknown by InterPro database scanning (Table 2 Suppl.). Furthermore, motif 8 and motif 12 were observed in members of subgroups IIa and IIb. Also, motif 5 and 10 were only found in some of the members in group III. These specific motifs seem to be important in the functional divergence of WRKY genes.

To recognize differentially expressed HvWRKYs under drought stress conditions at seedling and reproductive stages, four microarray datasets (GSE3170, GSE6990, GSE17669, and GSE15970) were investigated. Evaluating gene expression in barley seedlings of cv. Morex under different soil water content (SWC) (GSE6990) revealed that HvWRKY\_IId2, HvWRKY\_IId2, HvWRKY\_III23, HvWRKY\_IId5, and HvWRKY\_IId19 were significantly up-regulated under low soil water content. Further, HvWRKY\_

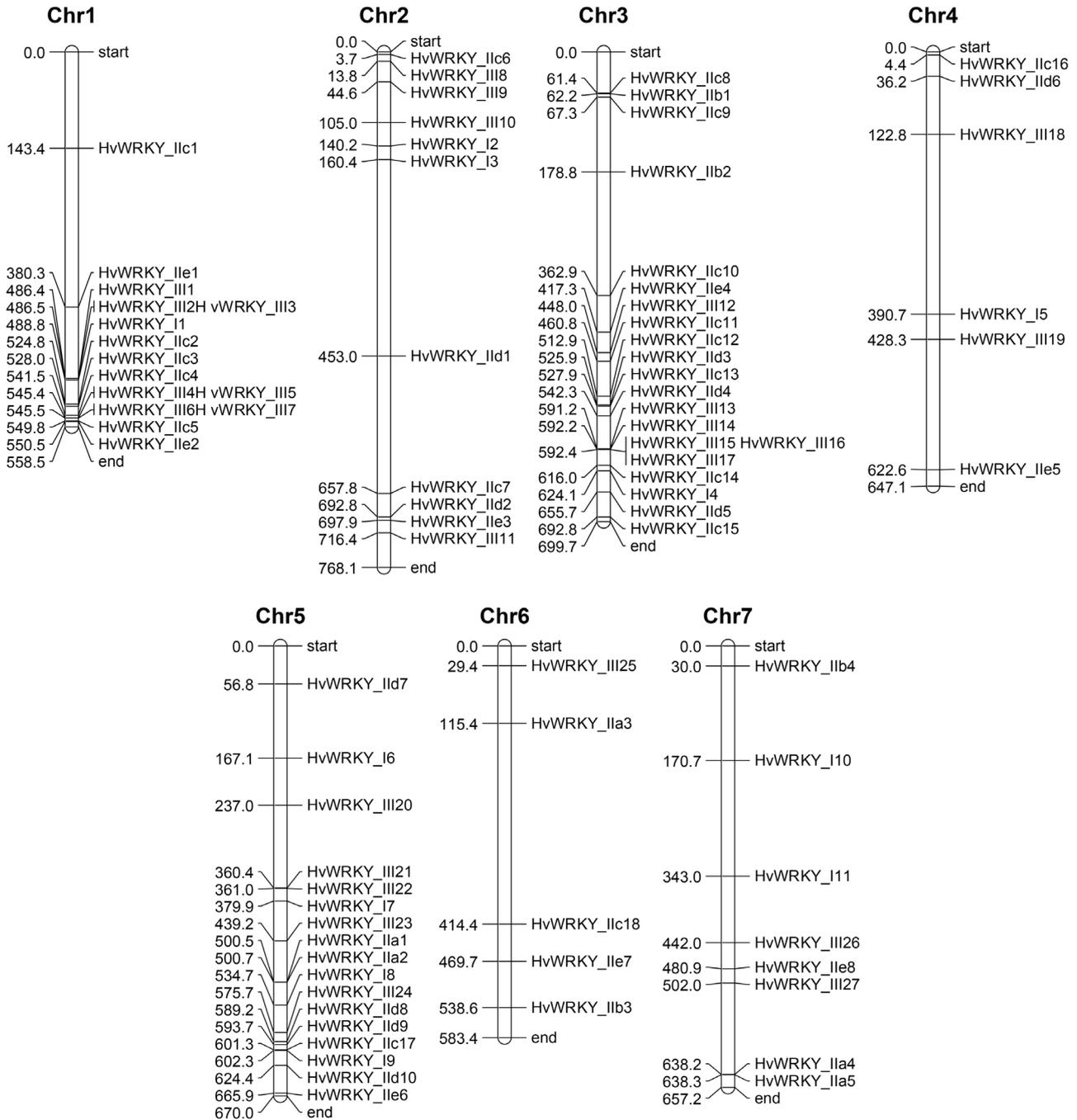


Fig. 2. Distribution of *HvWRKY* genes (83 out of 93) on the barley chromosomes (chromosomal distances are given in megabases). Chromosomal locations of 10 *HvWRKY* members are uncertain on the barley genome.

*I1b2* gene expression was up-regulated more when soil moisture declined gradually (Fig. 3). Comparisons between drought stress and control conditions at seedling stage in cv. Oregon Wolfe dominant and recessive genotypes indicated that the transcription of *HvWRKY\_I18* and *HvWRKY\_I1c19* were elevated under drought stress (Fig. 3). Differential gene expression analysis in two drought-tolerant and a drought-sensitive genotypes at reproductive stage under

drought stress revealed that *HvWRKY\_I1e3* was highly expressed in both drought-tolerant genotypes. Furthermore, the transcription of *HvWRKY\_III11* and *HvWRKY\_III7* grew in only one drought-tolerant genotype (Fig. 3). Finally, assessing differential gene expression on the photosynthetic organs of the barley spike (lemma, palea, and awn) and seed between drought and control conditions indicated that *HvWRKY\_I1b2* was the only up-regulated

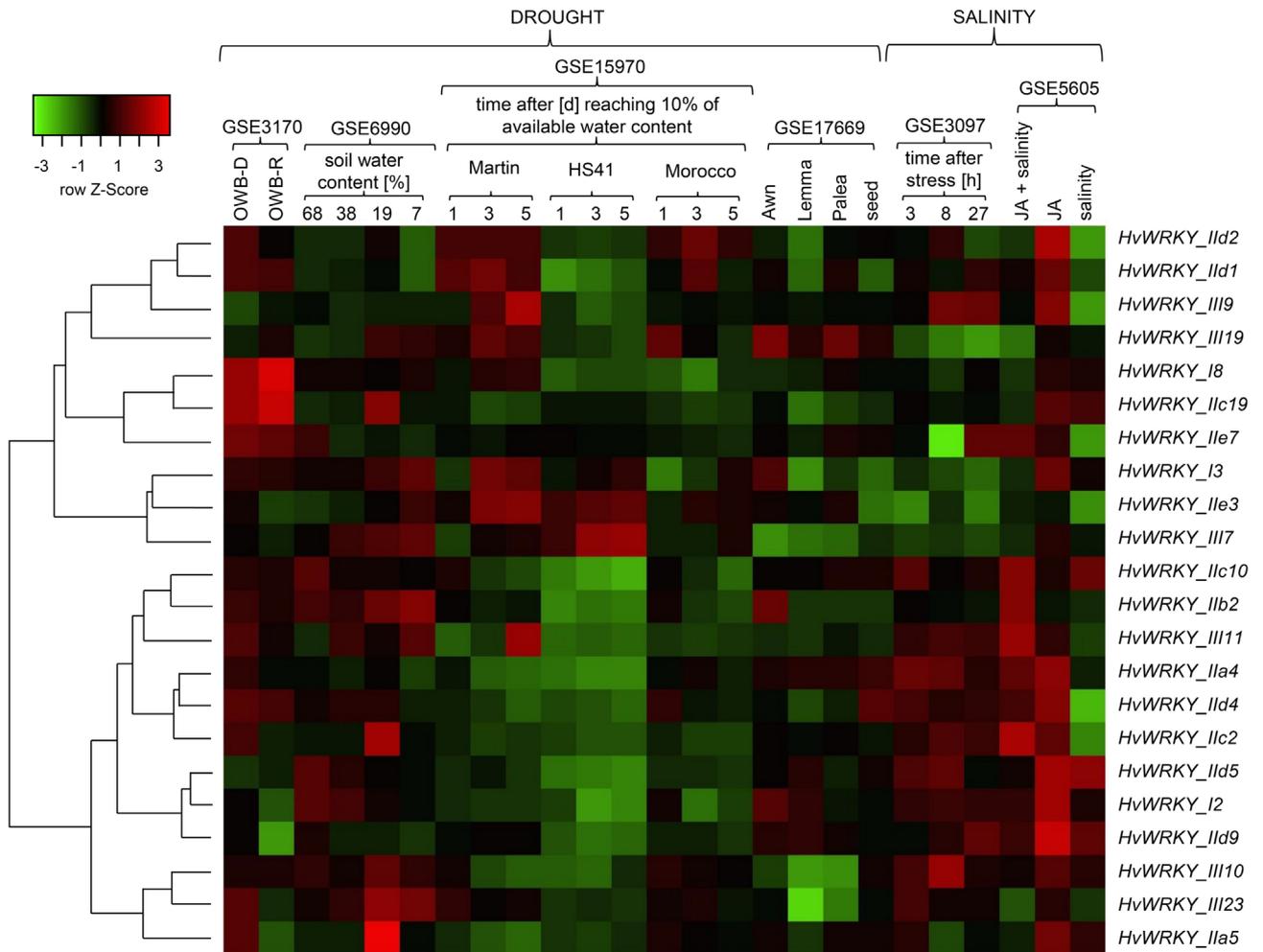


Fig. 3. Hierarchical clustering differential expression profiles of barley *WRKY* genes in 25 samples under drought and salinity stresses compared to optimum conditions.

member in the awn under drought (Fig. 3). Thus, it seems that *HvWRKY\_IId2*, *HvWRKY\_IId1*, *HvWRKY\_III9*, *HvWRKY\_III19*, *HvWRKY\_I8*, *HvWRKY\_IId19*, *HvWRKY\_IId7*, *HvWRKY\_I3*, *HvWRKY\_IId3*, *HvWRKY\_III7*, *HvWRKY\_IId10*, *HvWRKY\_IId2*, *HvWRKY\_III11*, *HvWRKY\_IId4*, *HvWRKY\_IId2*, *HvWRKY\_IId5*, *HvWRKY\_I2*, *HvWRKY\_IId9*, *HvWRKY\_III10*, *HvWRKY\_III23*, and *HvWRKY\_IId5* are involved in the response to drought stress at the seedling stage. On the other hand, *HvWRKY\_III7*, *HvWRKY\_IId3*, and *HvWRKY\_IId2* play a role in response to drought stress at the reproductive stage.

To investigate the *HvWRKY* gene expression patterns under salinity stress, three microarray datasets were considered. Studying differential gene expression at seedling stage under control conditions and salinity (GSE3097) with three time points indicated that *HvWRKY\_III10* was up-regulated under salinity (Fig. 3). According to a previous report (Walia *et al.* 2007), two kinds of genes with specific gene expression patterns would be good candidate genes to enhance the salinity tolerance: 1) genes with down-regulated expression under salinity stress while altered in pre-treatment with jasmonic acid (JA) followed by salinity; 2) genes which were up-regulated in JA pre-treatment followed by salinity stress which also responded to salinity and JA treatments as well. Therefore, we predict that *HvWRKY\_IId2*, *HvWRKY\_III11*, *HvWRKY\_IId2*,

and *HvWRKY\_IId4* may enhance salinity tolerance at the seedling stage (GSE5605) based on their gene expression patterns (Fig. 3).

Some *HvWRKY* genes (*HvWRKY\_IId2*, *HvWRKY\_IId1*, *HvWRKY\_III9*, *HvWRKY\_III19*, *HvWRKY\_I8*, *HvWRKY\_IId19*, *HvWRKY\_IId7*, *HvWRKY\_I3*, *HvWRKY\_IId3*, *HvWRKY\_III7*, *HvWRKY\_IId10*, *HvWRKY\_IId2*, *HvWRKY\_III11*, *HvWRKY\_IId4*, *HvWRKY\_IId2*, *HvWRKY\_IId5*, *HvWRKY\_I2*, *HvWRKY\_IId9*, *HvWRKY\_III10*, *HvWRKY\_III23*, and *HvWRKY\_IId5*) were selected based on the aforementioned microarray datasets analysis to confirm the gene expression patterns at seedling stage in drought-sensitive and tolerant genotypes under drought conditions by real-time PCR. Drought and salt stresses both cause cellular dehydration. Further, their signal transduction pathways have cross talk with each other (Bartels and Sunkar 2005, Miller *et al.* 2010). *HvWRKY\_III11* and *HvWRKY\_IId4* were significantly up-regulated in response to salinity *via* JA pathway. We hypothesized that they may play a role in response to drought stress; thus, we selected them for further investigation under drought conditions as well.

The phylogenetic analysis revealed that one (*HvWRKY\_I8*), one (*HvWRKY\_IId5*), one (*HvWRKY\_IId2*), two (*HvWRKY\_IId2* and *HvWRKY\_IId19*), one (*HvWRKY\_IId4*), and two (*HvWRKY\_III11* and *HvWRKY\_III10*)

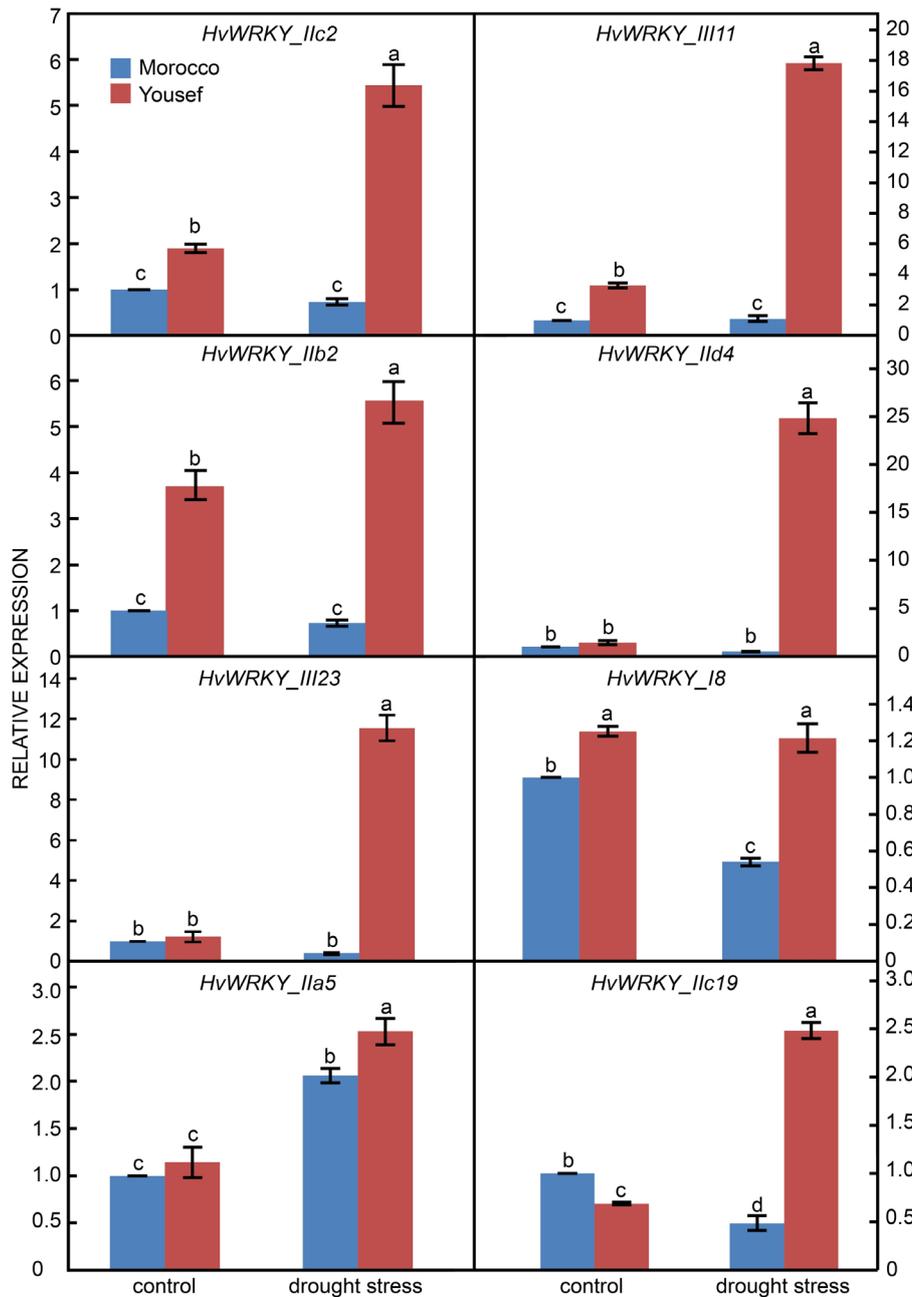


Fig. 4. Relative expression profiles of eight *HvWRKY* genes at the seedling stage with three independent biological replicates in drought-tolerant (Yousef) and drought-sensitive (Morocco) cultivars of barley under severe drought stress. Transcription in the sensitive genotype under water optimum conditions was used as a calibrator. Y-axis and error bars represent the fold change and standard deviation, respectively.

*III23*) candidate genes belong to the group I, IIa, IIb, IIc, IID, and III cluster, respectively (Fig. 2). Analysis of variance indicated that the effects of water treatments were significant on the relative expression of all eight *HvWRKY* genes ( $P \leq 0.01$ ) under both optimum and drought stress conditions (Table 3 Suppl1.). The expression of *HvWRKY\_IIC2*, *HvWRKY\_III11*, *HvWRKY\_IIB2*, *HvWRKY\_IID4*, and *HvWRKY\_III23* was significantly up-regulated in the tolerant cultivar (Yousef) while their expressions remained unchanged in the sensitive genotype (Morocco)

under drought stress. *HvWRKY\_IIA5* was significantly up-regulated in both genotypes under drought stress at the seedling stage though more in Yousef than in Morocco. *HvWRKY\_IIC19* was notably up-regulated in the tolerant (Yousef) genotype under severe drought stress, while it was down-regulated in the susceptible Morocco genotype. No significant change was found in the transcription of *HvWRKY\_I8* in Yousef, while it was reduced in Morocco under drought stress at the seedling stage (Fig. 4).

Based on the phylogenetic tree, *HvWRKY\_IIC2* and

*HvWRKY\_I1c19* were closely joined together, while *HvWRKY\_I1a5* gene was clustered with *HvWRKY\_I1a4*, *HvWRKY\_I1a3*, *AtWRKY18*, and *AtWRKY60* (Fig. 2). A previous report indicated that *HvWRKY\_I1c19* (*HvWRKY19*) and *HvWRKY\_I1a4* (*HvWRKY2*) are up-regulated in vegetative organs of the resistant genotype but not in the sensitive genotype of barley under drought (Li *et al.* 2014). Also, *HvWRKY\_I1a3* (*HvWRKY1/38*) is involved in response to abiotic stress including cold and drought stress at the seedling stage in barley via an ABA-independent way (Mar *et al.* 2004). *AtWRKY18* and *AtWRKY60* in the cluster are transcriptional activators in response to ABA and abiotic stresses such as drought and salinity in *Arabidopsis* wild type (Chen *et al.* 2010). Based on the obtained gene expression results (Fig. 4), the aforementioned microarray data (Fig. 3), and phylogenetic clustering (Fig. 2), *HvWRKY\_I1a5* and *HvWRKY\_I1c19* seem to be the transcriptional activator in response to drought-stress.

*HvWRKY\_III11* along with the *HvWRKY\_III27*, *AtWRKY54*, and *AtWRKY70* lie in the same group based on the phylogenetic tree (Fig. 2). The *wrky54/wrky70* double mutant reveals earlier leaf senescence compared to wild type in *Arabidopsis*. Thus, these two genes co-operate with each other and they are negative regulators in senescence (Besseau *et al.* 2012). In addition, *AtWRKY46*, *AtWRKY54*, and *AtWRKY70* are related to *OsWRKY47* in rice. Knock-out and overexpression of *OsWRKY47* lead to drought susceptibility and tolerance, respectively (Raineri *et al.* 2015). Overall, the obtained results suggest that *HvWRKY\_III11* might act as a negative regulator of leaf senescence, helping the genotype to survive under drought conditions.

## Conclusions

In the present study, 93 members of *WRKY* gene family were identified in barley, classified into three groups and seven subgroups based on unrooted phylogenetic tree in barley and *Arabidopsis*. Eight drought-responsive candidate *WRKY* genes were predicted based on analysis of available microarray datasets under drought and salinity at the seedling stage in barley. Furthermore, the gene expression patterns in tolerant and susceptible genotypes by qRT-PCR indicated that all of candidate genes (*HvWRKY\_I1c2*, *HvWRKY\_III11*, *HvWRKY\_I1b2*, *HvWRKY\_I1d4*, *HvWRKY\_III23*, *HvWRKY\_I1a5*, and *HvWRKY\_I1c19*) are involved in the response to the drought stress at the seedling stage in barley. The achieved results provided useful information regarding the *HvWRKY* gene family and its drought-responsive members for further functional analysis. They might also be beneficial for achieving drought tolerant cultivars through molecular breeding or genetic engineering.

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