Sorghum WRKY transcription factor *SbWRKY45* enhanced seed germination under drought stress in transgenic *Arabidopsis*

Elamin BAILLO[1,2,3,4,*] , **Muhammad Sajid HANIF**[1,2,3], **Ping XU**[1,2,3], **Zhengbin ZHANG**[1,2,3]

[1]Key Laboratory of Agricultural Water Resources, Hebei Laboratory of Agricultural Water Saving, Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, University of Chinese Academy of Sciences, Shijiazhuang, China
[2]University of Chinese Academy of Sciences, Beijing, China
[3]Innovation Academy for Seed Design, Chinese Academy of Sciences, Beijing, China
[4]Agricultural Research Corporation (ARC), Ministry of Agriculture, Gezira, Sudan

Abstract: The WRKY transcription factors (TFs) family is an important family of plant-specific TFs, playing vital roles in various abiotic and biotic stress responses. WRKY TFs are gaining considerable attention due to their significant roles in stress responses. However, their functions in sorghum (*Sorghum bicolor*) are lagging behind. In this study, a WRKY gene designated as *SbWRKY45* was isolated and characterized from sorghum. *SbWRKY45*, belonging to Group IIa, consists of one intron and two exons and encodes 430 amino acids. *SbWRKY45* is located in chromosome 4. The *cis*-element prediction analysis showed that the promoter region of *SbWRKY45* has several abiotic stress-associated elements. The qRT-PCR results showed that *SbWRKY45* was significantly up-regulated in response to drought and cold under salt treatments, was notably changed, and was induced weakly under heat stress. *SbWRKY45* exhibited a response to stress in different sorghum tissues, including leaves, stems, and roots. A tissue-specific expression pattern showed that *SbWRKY45* was highly expressed in roots compared with leaves and stems, suggesting that *SbWRKY45* may play an important role in roots. Overexpression of *SbWRKY45* increased germination rates and promoted root growth in transgenic *Arabidopsis* under drought stress. Taken together, our findings indicate that *SbWRKY45* may be involved in mediating the response to drought stress and play a vital role in the abiotic stress response of sorghum.

Key words: WRKY; sorghum, transcription factors, abiotic stress, gene, overexpression

1. Introduction

The WRKY is a plant-specific transcription factor (TF) family, which plays essential roles in plant responses to environmental stresses (Eulgem et al., 2000). The WRKY domain is about 60 amino acid residues in length, consisting of a highly conserved heptapeptide sequence WRKYGQK at the N-terminus and zinc-finger-like motif (C-X_{4-5}-C-X_{22-23}-H-X_1-H) or (C-X_7-C-X_{23}-H-X_1-C) at the C-terminus (Eulgem et al., 2000; Chen et al., 2019a). The conservation of the WRKY domain is determined by its cognate binding site. WRKY TFs bind to the *cis*-acting elements W box (TTGACC/T), by which WRKY TFs activate or repress the downstream genes. It was demonstrated that WRKY TFs play a vital role in regulating several plant processes, including regulation of transcriptional reprogramming associated with the plant stress response. WRKY TFs are diversely distributed in plants and respond to different abiotic and biotic stresses (Erpen et al., 2018). In the last few years, increasing genome sequencing data from diverse plant species with the development of novel sequencing technologies and bioinformatics provided an excellent platform for the genome-wide analysis of *WRKY* family genes. Tremendous progress has been made in this regard, and genome-wide identification and functional characterization of *WRKY* genes family have been done in several crop plants (Chen et al., 2019b). Recently, several *WRKY* genes have been identified, for example, 56 putative *WRKYs* have been found in tea, 49 in coffee, 70 in chickpea, and 61 in Chinese jujube (Wang et al., 2018; Dong et al., 2019; Waqas et al., 2019; Chen et al., 2019b).

Previous studies indicated that WRKY genes induced a response to abiotic stress and improved stress tolerance. For example, *ZmWRKY40*-overexpressing transgenic *Arabidopsis* exhibited significant drought tolerance, through controlling different processes, including the up-regulation of stress-associated genes and lowering reactive oxygen species (ROS), which led to enhancement of the activities of other process, including peroxide dismutase (POD) and

* Correspondence: aminomooon14@gmail.com

catalase (CAT) in response to drought stress treatment (Wang et al., 2018a). Furthermore, *ZmWRKY106* was induced by drought, heat, and exogenous ABA; transgenic *Arabidopsis* plants overexpressing *ZmWRKY106* showed improved tolerance to drought by regulating stress-related genes via the ABA-signaling pathway (Wang et al., 2018b). In wheat, *TaWRKY1* and *TaWRKY33* in wheat are other examples; both were involved in response to drought and heat stress by activating several stress-related downstream genes, promoted root growth, and increased germination rate in transgenic *Arabidopsis*; also, the transgenic lines showed a lower rate of water loss comparing to wild-type plants during hydration (He et al., 2016).

The functional characterization of many *WRKY* genes has been reported in *Arabidopsis*; however, in nonmodel plants, few studies have been reported. For instance, overexpression of *Arabidopsis WRKY30* exhibited enhanced drought and heat tolerance in transgenic wheat. Furthermore, the expression analysis showed up-regulation of stress-related genes and enzyme-encoding genes (El-Esawi et al., 2019). In cotton, overexpression of *GhWRKY33* in transgenic *Arabidopsis* plants exhibited reduced tolerance to drought stress and participation in ABA signaling pathway. Moreover, the transgenic lines showed increased expression of drought-related genes and ABA-responsive genes (Wang et al., 2019). Transgenic wheat plants overexpressing *AtWRKY30* showed enhanced heat and drought stress tolerance. The proline content, relative water content, soluble protein, soluble sugar content, and antioxidant enzymes such as peroxidase (POX) and superoxide dismutase (SOD) were increased in transgenic plants overexpressing *AtWRKY30* (El-Esawi et al., 2019). Previous studies have indicated that *WRKY* genes are involved in response to low temperature. For instance, *VaWRKY12* overexpression enhanced cold tolerance in transgenic *Arabidopsis* and grapevine plants. The antioxidant enzymes encoding genes (glutathione S-transferases and peroxidases) were up-regulated under cold stress treatment (Zhang et al., 2019). Moreover, overexpression of cucumber (*Cucumis sativus*) *WRKY* gene *CsWRKY46* enhanced seedling survival under freezing stress in transgenic *Arabidopsis* plants. Furthermore, higher proline accumulation was observed whereas less electrolyte leakage and lower MDA levels were found. The study concluded that cucumber *CcWRKY46* involved and enhanced cold stress tolerance in the transgenic plants (Zhang et al., 2016).

Sorghum (*Sorghum bicolor*) is the fifth most important cereal crop; although many WRKY TFs have been identified in various species such as *Arabidopsis*, maize, wheat, and rice, only a few or no functional studies have been reported in sorghum. Our previous report indicated that there were 94 *WRKY* genes in sorghum genome; to elucidate the roles of *SbWRKYs*, we found that *SbWRKY45*

was strongly induced by drought, and its homolog from maize *ZmWRKY40* improved drought tolerance when overexpressed in *Arabidopsis*; therefore, we wish to know whether a WRKY homolog in sorghum can function similarly as in maize; if so, the gene could be a candidate for further genetic modification and engineering in sorghum. In this study, we isolated and characterized *SbWRKY45* gene from sorghum and determined its response to abiotic stress. The overexpression of *SbWRKY45* conferred drought tolerance and enhanced seed germination in *Arabidopsis*.

2. Materials and methods
RNA was extracted from 2-week-old sorghum seedlings using TRIzol reagent (TransGen Biotech, Beijing, China) following the manufacturer's instructions. The DNA was removed using RNase-free DNase I (TransGen Biotech, Beijing, China); then, the RNA concentration was checked using a ThermoScientific NanoDrop^MT 2000c Spectrophotometer. The first-strand cDNA was synthesized from 1 μg of total RNA using First-Strand Synthesis following the manufacturer's protocol (TransGen Biotech, Beijing, China) and used as the template for PCR reaction.

2.1. Cloning of SbWRKY45 and sequence analysis
The primers of *SbWRKY45* (XM_002451621.1), also known as (SORBI_3004G065900), was designed based on the sequence obtained from NCBI (https://www.ncbi.nlm. gov/ 11/9/2019) to clone the gene from sorghum. The PCR product was cloned into a pMD18-T vector (TAKARA BIO, Beijing, China) and sequenced. The sequence of SbWRKY45 was analyzed using blast search, and other orthologs were obtained from the NCBI database. Multiple sequence alignment and phylogenetic tree analysis were performed using MEGA7.0 with neighbor-joining (NJ) method using default values for all the parameter, and the confidence levels were estimated with bootstrap analysis of 1000 replicates (Kumar et al., 2016). The functional domain of *SbWRKY45* was predicated by SMART (http://coot. embl-heidelberg.de/SMART/ 16/10/2019) (Letunic and Bork, 2018). The protein structure analysis was conducted by (http://genome.cbs.dtu.dk/services/CPHmodels-2_0Server-3D.htm, 16/10/2019). The *cis*-acting elements in the promoter region of *SbWRKY45* were predicted using plant *cis*-acting regulatory element (PlantCARE, http:// bioinformatics.psb.ugent.be/webtools/plantcare/html, 18/4/2020) as described by (Gao et al., 2018). Additionally, the gene structure of *SbWRKY45* was identified by using the Gene Structure Display Server GSDS2.0 (Hu et al., 2015).

2.2. Stress treatment and samples collection
Seeds of *Sorghum bicolor* genotype (SX44B) used in this study were provided by Professor Zhang Fuyao (Sorghum

Institute, Shanxi Academy of Agricultural Sciences, Shanxi, China). The sorghum seeds were surface-sterilized with 5% sodium hypochlorite (*v/v*) for 1–2 min then rinsed three times with distilled water. The sterilized seeds were germinated on two layers of wetted paper and incubated at 25 °C in dark for 3 days. The seedlings were transferred to pots containing a soil mixture of vermiculite and peat moss at the ratio of 1:1. The seedlings were grown in a growth chamber with 60%–70% relative humidity, 28/23 °C day/night, and 16-h light/8-h dark photoperiod. The seedlings were grown under normal conditions for 2 weeks and then used for different abiotic stress (drought, cold, heat, and salt) treatments. For drought treatment, seedlings were treated with PEG6000 20% (Osmolovskaya et al., 2018). For cold and heat, the seedlings were exposed to 4 °C and 40 °C, respectively. For salinity stress, the potted seedlings were saturated with 250 mM NaCl solution (Zhao et al., 2019). The seedling samples were collected at 0, 3, 6, 12, and 24 h, and different tissue samples such as leaves, stems, and roots were collected. All samples were frozen in liquid nitrogen and stored at −80 °C until being used for RNA extraction.

2.3. Quantitative real-time PCR analysis

Total RNA was extracted from sorghum tissues based on the protocol described (Promega, China). The quantitative real-time PCR (qRT-PCR) was performed using the 7500 Real-time PCR system. The qRT-PCR primers used in this study are listed in Table S1. *TransStart* Green qPCR Supermix UDG Kit (TransGen Biotech, Beijing, China) was used, and all the qPCR analysis were conducted in a 20 μL reaction mixture, containing 0.4 mM of gene-specific primers, 10 μL of super mix, and 1 μL of cDNA template. The qRT-PCR procedures were performed as follows: 95 °C for 10 min, 40 cycles of 94 for 5 s, 60 °C for 15 s, and 72 °C for 10 s. In quantitative analysis, the sorghum *actin* gene was used as an internal control. The Ct values methods (ΔΔCT) were used for quantification analysis (Livak and Schmittgen, 2001). The data were analyzed according to method described by (Le et al., 2011).

2.4. Construction of the expression vector and generation of transgenic Arabidopsis

The CDS of SbWRKY45 was amplified using the forward primer 5′CACTGTTGA TA<u>CATATG</u>ACCTTCT CTGAG CCTTGACC3′ and the reverse primer 5′ATTCAGAATT<u>G AGCTC</u>GTCGTCCCTTG TCAGCGTCATCG3 (NdeI and SacI site underlines, respectively). The fragment of SbWRKY45 was ligated into pRI201vector, which was digested with NdeI and SacI under the control of the Cauliflower mosaic virus (CaMV) 35S promoter with kanamycin as the selectable marker. The recombinant plasmid was confirmed by sequencing. The recombinant vector pRI201-SbWRKY45 was introduced into *Agrobacterium tumefaciens* EHA 105 for *Arabidopsis*

transformation using the floral dip method (Niu et al., 2012).

2.5. Drought tolerance analysis of SbWRKY45 transgenic Arabidopsis plants

Further investigation of the biological functions of transgenic *Arabidopsis* plants overexpressing *SbWRKY45* was created. Germination and growth assay were conducted to identify the phenotype differences between wild-type and transgenic lines under drought stress. The germination assay was performed by culturing the seed of transgenic lines and wild type on 1/2MS basal medium plates supplemented with or without 4% PEG6000. The media plates were incubated at 4 °C for 3 days and then moved to 22 °C with a photoperiod of 16 h light/8 h dark. Seeds were considered to be germinated when radicals emerged from the seed coat. For root growth assay, 1-week-old T2 transgenic *Arabidopsis* lines grown on the 1/2MS medium were transferred to 1/2MS media supplemented with or without 8% PEG6000 and kept in growth chamber vertically. The root lengths were photographed and measured (Ma et al., 2014).

2.6. Statistical analysis

In this study, three biological replicates were used throughout the experiment. When appropriate, the analysis of variance (ANOVA) was used for quantitative real-time PCR to determine the statistical significance of the differential expression patterns between treatments and/or between tissues. For the germination and growth assay statistical analysis was performed using Student's *t* test to determine the statistical differences relative to WT. Mean values of three independent experiments were used to plot figures, the error bars represent the standard deviation.

3. Results

3.1. Gene cloning of the SbWRKY45 and sequencing

SbWRKY45 with (accession number: XM_002451621.2) was successfully isolated from sorghum using reverse transcription PCR (RT_PCR), the gene designated as *SbWRKY45*. The gene selected based on the similarity to the ortholog counterpart *ZmWRKY40* (GRMZM2G120320) in maize was induced by abiotic stress and improved drought stress tolerance in transgenic plants (Wang et al., 2018a); therefore, we are interested to know whether the ortholog from sorghum can have the same role in response to drought stress. The *SbWRKY45* gene was amplified with gene-specific primers; the sequence analysis of *SbWRKY45* gene presented that the gene was 1040 bp in length and coded a deduced protein sequence of *SbWRKY45* containing about 340 amino acid residues along with a predicted molecular weight (MW) of 38.9 and theoretical isoelectric point (PI) 8.45.

The SbWRKY45 protein domains analysis confirmed that *SbWRKY45* belongs to group IIa of the WRKY family in sorghum, according to our classification based on the method used by (Eulgem et al., 2000). Furthermore, the results showed that SbWRKY45 contained WRKYGQK domain, which is 60 amino acid residues at the N-terminus, and contained an extended zinc finger motif (C-X_5-C-X_{23}-H-X_1-H) at the C-terminus compared with the typical WRKY domain as in *Arabidopsis* WRKY. There are 10 chromosomes in the sorghum genome, and *SbWRKY45* is located in the long arm of chromosome 4 with one copy (Baillo et al., 2020).

The protein domain analysis showed that *SbWRKY45* has WRKY domain and zinc finger domain. Sequence alignment showed that *SbWRKY45* is highly similar to orthologous genes, including *Zea mays ZmWRKY40* (GRMZM2G120320), *Arabidopsis thaliana AtWRKY40* (AT1G80840), *Setaria italica SiWRKY71* (XP_004951681.1), Pm *Panicum miliaceum WRKY40* (RLM80886.1), and *Triticum aestivum TaWRKY80* (AFW98256.1) (Figure 1). All sequences used were found to have highly conserved domain WRKYGQK sequence as well as nucleus localization signal (NLS).

3.2. Phylogenetic relationship of SbWRKY45 with other WRKY transcription factors

For the phylogenetic analysis of *SbWRKY45*, similar protein members from other species were retrieved and used. Eighteen available sequences from different species with high similarity were downloaded from NCBI. SbWRKY45 was classified in one clade with ZmWRKY40, ZmWRKY71, AtWRKY40, and SsWRKY; all members shared the highest genetic similarities, suggesting a possible functional similarity (Figure 2). Furthermore, phylogenetic analysis showed some divergence of mono and dicots orthologous genes.

3.3. Structure and promoter analysis of SbWRKY45

The structure analysis conducted by using Gene Structure Display Server (GSDS2.0) revealed that *SbWRKY45* contained two exons and one intron besides untranslated region upstream. To detect the abiotic stress that induces *SbWRKY45* and elucidate the underlying biological functions of the gene, 1000 bp of the upstream was used to predict the cis-acting element in the promoter region of *SbWRKY45* using online cis-element prediction software PlantCARE. We found that the promoter region of *SbWRKY45* has several abiotic stress-associated elements such as abscisic acid-responsive element (ABRE), TGACG-motif, light-responsive element (C-box), MYB, and W-box, which are binding sites for *WRKYs* that might indicate *SbWRKY45* being involved in WRKY transcription factor interactions that activate or deactivate stress-related genes (Table 1). A four-stranded B-sheet with a zinc-binding pocket was formed by conserved Cys/His residues in the

WRKY domain in the structure of *SbWRKY45*.

3.4. Expression of SbWRKY45 gene under abiotic stresses and different tissues

The expression patterns of *SbWRKY45* under various abiotic stresses and different tissues were investigated by quantitative real-time PCR (qRT-PCR). The results showed that *SbERKY45* was differentially induced by drought, salt, and cold but was induced weakly by heat stress treatment (Figure 3). *SbWRKY45* expression was induced gradually to reach a maximum at 6 h with 12.2-fold change after drought treatment and then declined to a level similar to the control (Figure 3A). In tissue-specific expression, *SbWRKY45* was highly induced within 6 h in roots in response to drought, but the gene was lower in leaves in comparison to other tested tissues. When treated with cold stress, the transcript of *SbWRKY45* was induced within 3 h, reached its peak (5.45-fold) at 6 h (Figure 3B), and decreased gradually after 12 and 24 h. *SbWRKY45* exhibited the highest response in roots in comparison with leaves and stems in a cold condition. Under slat treatment, the transcript of *SbWRKY45* began significantly increasing after 3 h treatment (1.12-fold) and 6 h (1.14-fold) compared to the control (0 h) (Figure 3C). The highest expression level was recorded at 6 h in all tested tissues; however, in leaves the gene was stable at different time points and then decreased after 24 h. When treated with heat stress, the expression of *SbWRKY45* was down-regulated, although a slight increase was observed in leaves at 3 h (Figure 3D). *SbWRKY45* had a low response to heat stress compared with drought, cold, and salt stress. *SbWRKY45* was found to be differentially expressed in leaves, stems, and roots; however, *SbWRKY45* was more abundant in roots than that in leaves and stems, especially under drought and cold. The results revealed that the transcript level of *SbWRKY45* was increased gradually in roots under all treatments.

The expression pattern of *SbWRKY45* under abiotic stress was similar to its ortholog *ZmWRKY40* (Wang et al., 2018a), in particular under drought stress; however, they showed different expression patterns under cold and salt stress, and the time point for the begging expression was different. Furthermore, the expression pattern of tested tissues was similar and the highest expression was in root. These indicated that *SbWRKY45* and *ZmWRKY40* might play a similar role in response to abiotic stress, especially drought. These results suggested that the *SbWRKY45* may play a fundamental role in response to different abiotic stress.

3.5. Overexpression of SbWRKY45 improved seed germination under drought stress in transgenic Arabidopsis

The expression analysis showed that the *SbWRKY45* expression was increased significantly by drought; therefore, to further investigate the biological function of
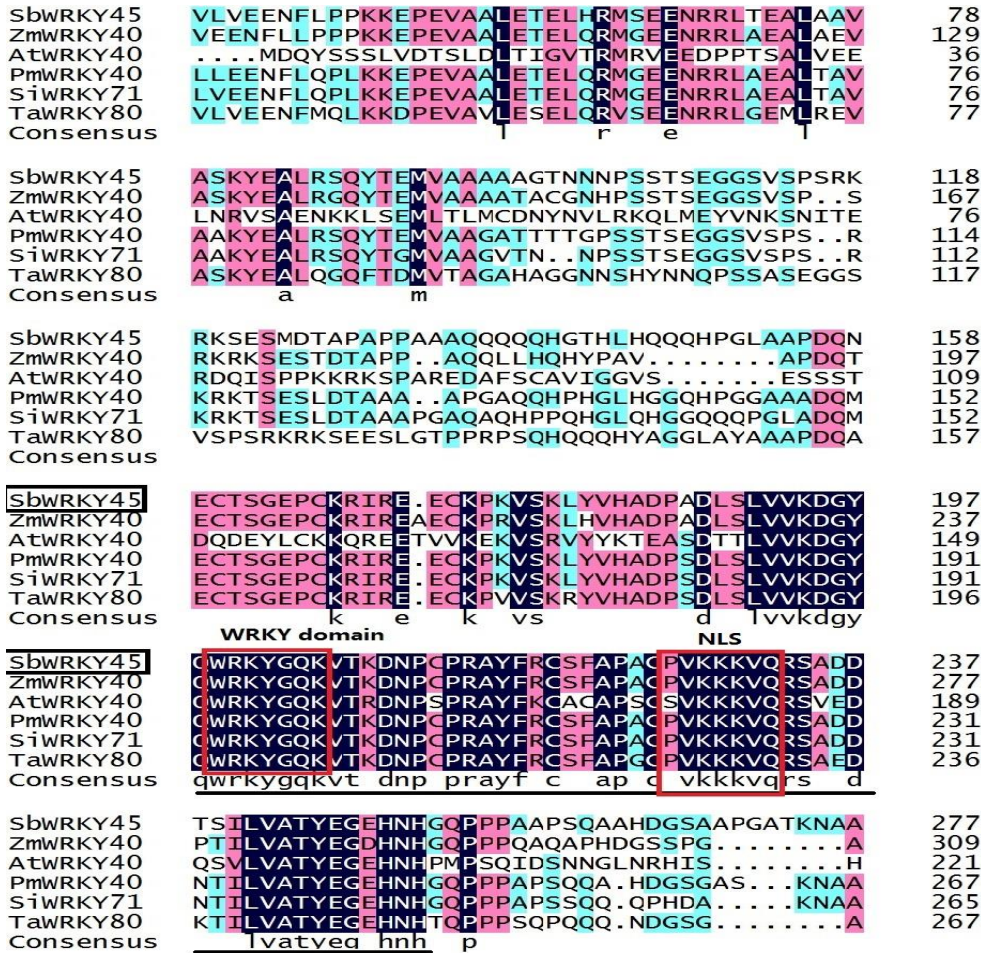
```
SbWRKY45    VLVEENFLPPKKEPEVAALETELHRMSEENRRLTEALAAV    78
ZmWRKY40    VEENFLLPPPKKEPEVAALETELQRMGEENRRLAEALAEV    129
AtWRKY40    ....MDQYSSSLVDTSLDLTIGVTRMRVEEDPPTSALVEE    36
PmWRKY40    LLEENFLQPLKKEPEVAALETELQRMGEENRRLAEALTAV    76
SiWRKY71    LVEENFLQPLKKEPEVAALETELQRMGEENRRLAEALTAV    76
TaWRKY80    VLVEENFMQLKKDPEVAVLESELQRVSEENRRLGEMLREV    77
Consensus                    l     r    e      l

SbWRKY45    ASKYEALRSQYTEMVAAAAAGTNNNPSSTSEGGSVSPSRK    118
ZmWRKY40    ASKYEALRGQYTEMVAAAATACGNHPSSTSEGGSVSP..S    167
AtWRKY40    LNRVSAENKKLSEMLTLMCDNYNVLRKQLMEYVNKSNITE    76
PmWRKY40    AAKYEALRSQYTEMVAAGATTTTGPSSTSEGGSVSPS..R    114
SiWRKY71    AAKYEALRSQYTGMVAAGVTN..NPSSTSEGGSVSPS..R    112
TaWRKY80    ASKYEALQGQFTDMVTAGAHAGGNNSHYNNQPSSASEGGS    117
Consensus           a       m

SbWRKY45    RKSESMDTAPAPPAAAQQQQQHGTHLHQQQHPGLAAPDQN    158
ZmWRKY40    RKRKSESTDTAPP..AQQLLHQHYPAV........APDQT    197
AtWRKY40    RDQISPPKKRKSPAREDAFSCAVIGGVS........ESSST   109
PmWRKY40    KRKTSESLDTAAA..APGAQQHPHGLHGGQHPGGAAADQM    152
SiWRKY71    KRKTSESLDTAAAPGAQAQHPPQHGLQHGGQQQPGLADQM    152
TaWRKY80    VSPSRKRKSEESLGTPPRPSQHQQQHYAGGLAYAAAPDQA    157
Consensus

SbWRKY45    ECTSGEPCKRIRE.ECKPKVSKLYVHADPADLSLVVKDGY    197
ZmWRKY40    ECTSGEPCKRIREAECKPRVSKLHVHADPADLSLVVKDGY    237
AtWRKY40    DQDEYLCKKQREETVVKEKVSRVYYKTEASDTTLVVKDGY    149
PmWRKY40    ECTSGEPCKRIRE.ECKPKVSKLYVHADPSDLSLVVKDGY    191
SiWRKY71    ECTSGEPCKRIRE.ECKPKVSKLYVHADPSDLSLVVKDGY    191
TaWRKY80    ECTSGEPCKRIRE.ECKPVVSKRYVHADPSDLSLVVKDGY    196
Consensus              k   e    k  vs         d   lvvkdgy
                 WRKY domain                          NLS

SbWRKY45    QWRKYGQKVTKDNPCPRAYFRCSFAPACPVKKKVQRSADD    237
ZmWRKY40    QWRKYGQKVTKDNPCPRAYFRCSFAPACPVKKKVQRSADD    277
AtWRKY40    QWRKYGQKVTRDNPSPRAYFKCACAPSCSVKKKVQRSVED    189
PmWRKY40    QWRKYGQKVTKDNPCPRAYFRCSFAPACPVKKKVQRSADD    231
SiWRKY71    QWRKYGQKVTKDNPCPRAYFRCSFAPACPVKKKVQRSADD    231
TaWRKY80    QWRKYGQKVTKDNPCPRAYFRCSFAPGCPVKKKVQRSAED    236
Consensus   qwrkygqkvt dnp prayf c  ap c  vkkkvqrs  d

SbWRKY45    TSILVATYEGEHNHGQPPPAAPSQAAHDGSAAPGATKNAA    277
ZmWRKY40    PTILVATYEGDHNHGQPPPQAQAPHDGSSPG........A    309
AtWRKY40    QSVLVATYEGEHNHPMPSQIDSNNGLNRHIS........H    221
PmWRKY40    NTILVATYEGEHNHGQPPPAPSQQA.HDGSGAS...KNAA    267
SiWRKY71    NTILVATYEGEHNHGQPPPAPSSQQ.QPHDA.....KNAA    265
TaWRKY80    KTILVATYEGEHNHTQPPSQPQQQ.NDGSG........A    267
Consensus      lvatyeg hnh  p
```

Figure 1. The WRKY domain features and sequence alignment. Alignment of a putative amino acid sequence of SbWRKY45 with its identified homolog from the identical amino is shaded in dark color. The WRKY DNA-binding domain, which is 60-amino acid marked by bold underline, also included the cysteine and histidine residues of putative zinc finger motif. The highly conserved domain WRKYGQK sequence and the nucleus localization signal (NLS) are highlighted by red boxes. *SbWRKY45* is enclosed in black boxes.

SbWRKY45, transgenic *Arabidopsis* plants overexpressing-*SbWRKY45* under the control of cauliflower mosaic virus 35S promoter were generated. From the transformation, we obtained 51 independent T_0 lines; specific primers were used for positive lines screening. The qRT-PCR analysis results of T_0 showed that among six *Arabidopsis* transformants lines (L2, L3, L7, L9, L20, and L39) overexpressing *SbWRKY45*, two lines (L2 and L3) revealed high *SbWRKY45* expression level; therefore, they were selected for further study after confirmation of the positive transgenic plants and their transcription level were advanced for a further generation to select T_2. The RT-PCR result conferred that the exogenous *SbWRKY45* was successfully expressed in transgenic *Arabidopsis*. Two lines of the T2 transgenic *Arabidopsis* overexpressing-*SbWRKY45* were selected for germination analysis. The seed germination rate of the *SbWRKY45*-overexpressing *Arabidopsis* and wild-type (WT) were observed in normal MS medium (control) and MS medium supplemented with 4% PEG6000 for drought stress. The germination rate of the transgenic *Arabidopsis* plants overexpressing-*SbWRKY45* was significantly higher than WT (Figure 4). Radical emergence was scored daily until no further germination occurred; the transgenic plants germinated better at each time point after being transferred to drought stress condition (MS + PEG) (Figure 4). When under normal condition (MS without PEG), there was no visible morphological, developmental, or cotyledon greening differences of the transgenic *Arabidopsis* plants compared with the WT.

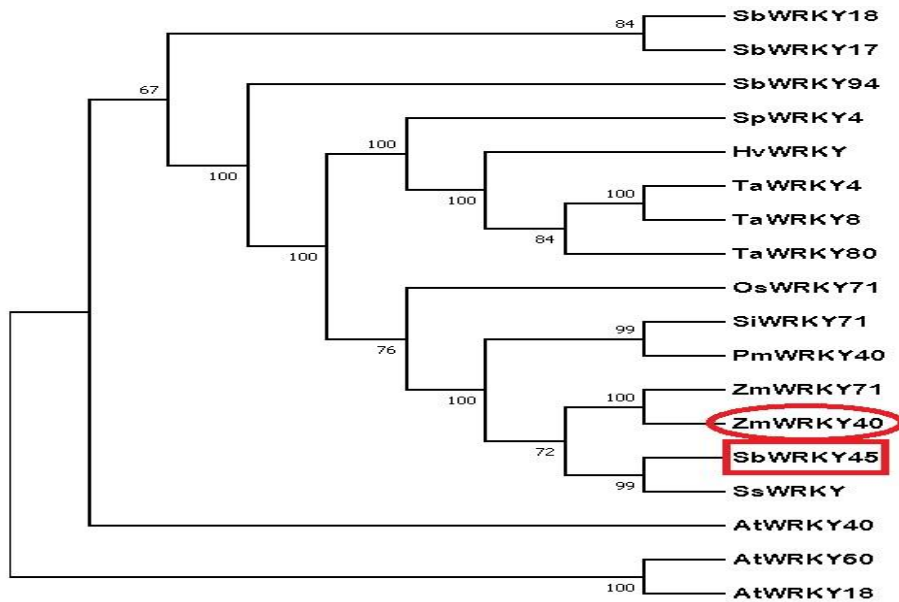*SbWRKY45*-overexpressing *Arabidopsis* plants were observed to have higher greening degree comparing

Figure 2. Phylogenetic tree of *SbWRKY45*, and phylogenetic relationship between SbWRKY45 and orthologous from different species**.**

Table 1. The putative *cis*-acting element of promoter *SbWRKY45*.

Cis-element	**Sequence**	**Number**	**Position**	**Function**
A-box	CCGTCC	4	581−	*Cis*-acting regulatory element
ABRE	ACGTG	2	541+	ABA and drought-responsive element
C-box	ACGAGCACCGCC	1	1034−	Regulatory element involved in light response
W-box	TTGACG	2	575−	SA-responsive element
CGTCA-motif	CGTCA	4	677+	MeJA responsive element
Motif I	GTACGTGGCG	2	725−	Root-specific regulatory element
MYB	CAACCA	1	746+	ABA and drought-responsive element

to WT (Figure 4) under drought stress; however, some transgenic plants have shown up-normal germination. The plants were photographed 2 weeks after transfer. The total root lengths of the transgenic and WT seedlings were measured after growing 7-day-old seedlings on 1/2 MS medium supplemented with or without 8% PEG6000 for 2 weeks. No differences have been observed in total root length under normal conditions; however, under drought stress, the root length of the transgenic line was slightly higher than WT. The seedling's growth was observed, and the 7-day-old seedlings of the transgenic *Arabidopsis* plants and WT were transferred to the MS supplemented with 8% PEG. Figure 5 shows that inhibition of root elongation by PEG stress was aggravated in WT plants in comparison to transgenic plants. The total root length of WT was one third that of transgenic *Arabidopsis* overexpressing *SbWRKY45*. The transgenic *Arabidopsis* plants overexpressing-

SbWRKY45 showed slight differences compared to wild-type; the plants were somewhat greener than WT under PEG treatment and the root was observed to be slightly longer than WT, suggesting that the *SbWRKY45* transgenic lines can tolerate drought stress treatment.

4. Discussion
Transcription factors such as NAC, bZIP, and WRKY are involved in response to different environmental stresses due to their major roles in plant growth and physiology (Ali et al., 2018; Lata et al., 2011; Baloglu et al., 2014). Among TF, WRKY TF has played an important role in the regulation of transcription reprogramming related to plant abiotic stress responses (Baillo et al., 2019). In sorghum, 94 *WRKY* genes provided basic information for functional studies of *WRKYs* in sorghum (Baillo et al., 2020). In the current study, it was elucidated that the
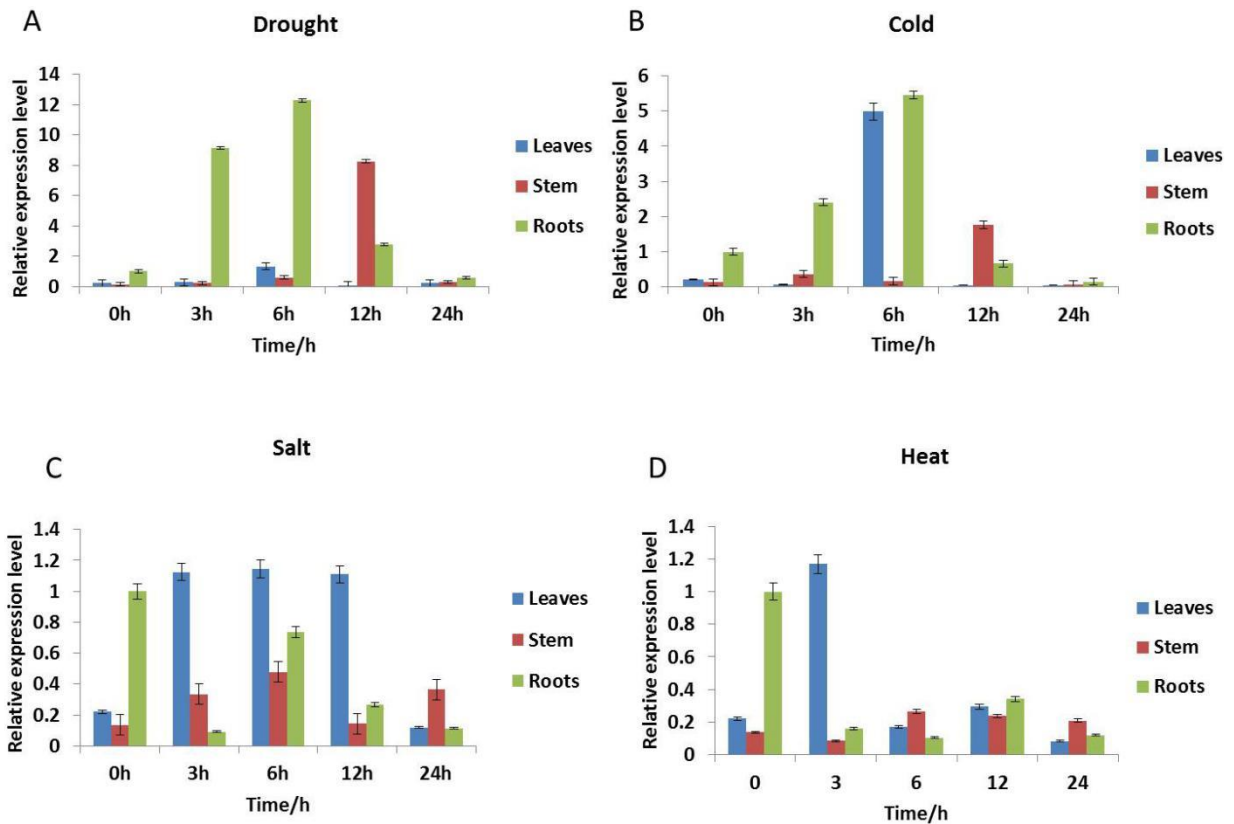
Figure 3. Expression analysis of *SbWRKY45* under drought, cold, salt, and heat, in different tissues. (**A**) Drought treatment (PEG6000), (**B**) cold treatment (4 °C), (**C**) NaCl treatment (250 mM), and (**D**) heat treatment (40 °C). The horizontal ordinate represents samples that were collected at 0 h, 3 h, 6 h, 12 h, and 24 h. The vertical represents fold change, and the colored ordinates represent different tissues. Data represent means and standard errors of three replicates.

functions of *SbWRKY45* in response to abiotic stresses in particular drought. *SbWRKY45* was the ortholog of maize *ZmWRKY40,* which has improved drought stress tolerance in *Arabidopsis* and provided the basis for cloning functional *WRKY* genes.

Our results confirmed that *SbWRKY45* is a member of sorghum IIa subgroup according to the classification of *WRKYs*. Sequence analysis of the *SbWRKY45* transcription factor revealed it consists of one WRKY domain and a C_2H_2 zinc finger motif. Generally, WRKY TFs consist of WRKY domain and C_2H_2 or C_2HC zinc-finger motif, which is used for WRKY TFs classification in all plant species (Chen et al., 2012). The structure of *SbWRKY45* was similar to *ZmWRKY40* and *AtWRKY40*; *ZmWRKY40* was isolated from maize and conferred improved drought tolerance in *Arabidopsis* (Wang et al., 2018a).

The sequence alignment and phylogenetic analysis demonstrated that *SbWRKY45* was clustered into IIa subgroup, which was more closely related to *ZmWRKY40*, *SsWRKY*, and *AtWRKY40*. The results indicated that *SbWKY45* is an important member of the WRKY TF family in sorghum. The predicted *cis*-element was found

to contain several stress response elements besides W-box, such as ABRE (ABA and drought-responsive elements), the CGTCA motif MeJA, MYB (ABA and drought-responsive elements), and C-box (regulatory element involved in light response). All these elements may play a vital role in helping the promoter drive the expression of several downstream genes (Jiang et al., 2017). It is worth mentioning that WRKYs family members have been reported to bind directly to the W-box in the distance in the promoter of the target genes to activate or suppress the response to the stresses (Ciolkowski et al., 2008). The findings above suggest that *SbWRKY45* might activate the expression of the stress-responsive genes via binding to the W-box in their promoter region.

Previous studies reported that *WRKY* genes can be induced by several abiotic stresses (Chen et al., 2019b). The expression of *SbWRKY45* under drought, cold, salt, and heat was enriched mostly in the roots compared with the leaves and stems. Under drought and cold stress treatments, the transcript level of *SbWRKY45* in roots was remarkably increased, especially in drought and was the highest among all tested tissues (Figure 3A). It has been
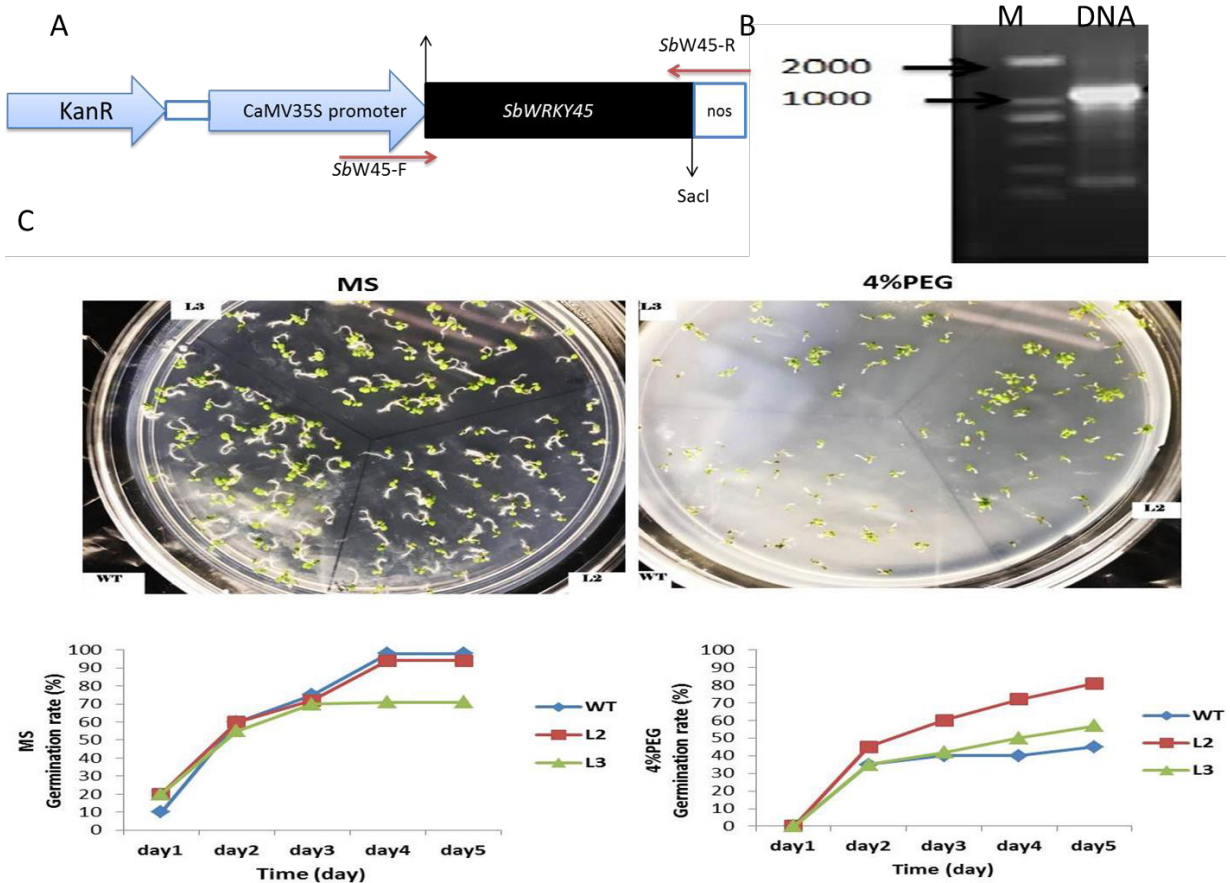
Figure 4. Characterization of transgenic *Arabidopsis*. (**A**) Schematic diagram of the transformation vector pRI201-*SbWRKY45*, (**B**) The 1040 bp of *SbWRKY45*, (**C**) Germination rates of the transgenic *Arabidopsis* overexpressing *SbWRKY45* lines (line2 and line3) and WT.

reported that sorghum roots showed a larger number of differentially expressed genes in response to drought. The authors highlighted WRKY transcription factors earlier as representing the majority of genes under drought (Varoquaux et al., 2019). This finding supports our results, which showed that the expression of *SbWRKY45* was higher in the roots under drought stress conditions. Furthermore, previous studies have proved that various WRKYs were induced in response to drought in several crops, for example, several *WRKY* genes were reported in maize (Kimotho et al., 2019). Additionally, under cold stress treatment, *SbWRKY45* showed increased expression level. Our results are inconsistent with previous studies, which confirmed the involvement of *WRKYs* in cold stress. For example, in rice, *OsWRKY71* showed increased expression level and had a positive function in cold tolerance by regulating downstream genes (Kim et al., 2016). Likewise, in salt, the expression level of *SbWRKY45* was up-regulated; however, it was less expressed in comparison with the expression under drought and cold. A similar result was found in maize, where *ZmWRKY33* was induced by high

salt treatment. Additionally, exogenous expression of *ZmWRKY33* in *Arabidopsis* improved salt stress tolerance under stress conditions (Li et al., 2013). Interestingly, in this study, *SbWRKY45* under heat stress condition was found to be down-regulated. However, this result disagrees with previous results; for example, *TaWRKY1* and *TaWRKY33* from wheat were induced by heat stress and confer heat resistance in transgenic *Arabidopsis* plants (He et al., 2016). We mentioned many pieces of evidence that *SbWRKY45* was involved in a positive response to stress. However, *SbWRKY45* was reported to have the opposite function in response to pathogen infection, for example, the expression of *SbWRKY45* was repressed during red stripe disease infection caused by *Herbaspirillum rubrisubalbicans* in sorghum (Tuleski et al., 2020). The repression was attributed to the bacteria's ability to modulate plant immunity. Moreover, the orthologous gene of *SbWRKY45* in melon (*CmWRKY15*) was down-regulated in response to powdery mildew infection, implying that *SbWRKY45* might play negative roles in response to pathogens (Jiao et al., 2018). Moreover,
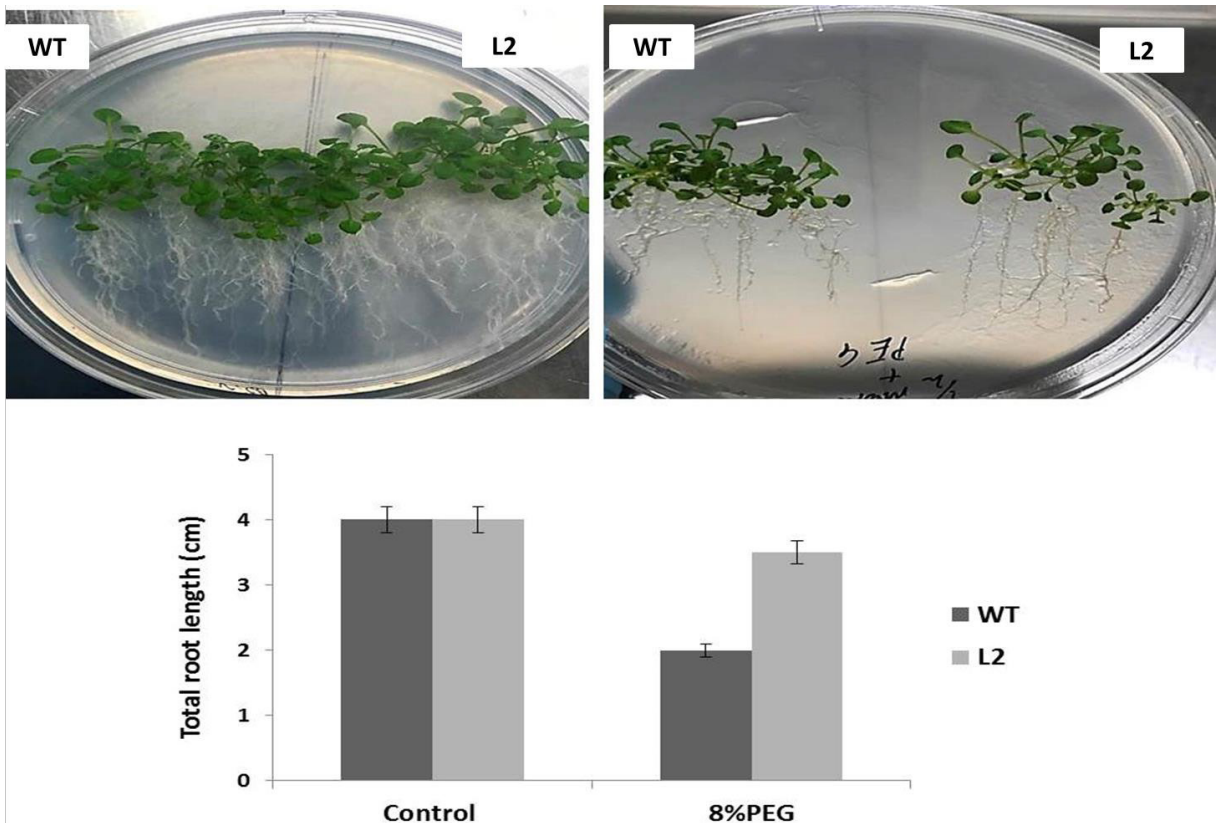
Figure 5. Phenotype of *SbWRKY45* transgenic *Arabidopsis* under drought treatment. The root length of the transgenic lines and WT. The data represent the means ± SDs of three independent biological replicates.

SbWRKY45 orthologous in Arabidopsis *AtWRKY18* and *AtWRKY40* negatively regulate the expression of defense response genes which enhanced the pathogen invasion (Brotman et al., 2013). Furthermore, *AtWRKY18 and AtWRKY40* both negatively regulate *Golovinomyces orontii* infection (Schön et al., 2013). These orthologous studies suggest that *SbWRKY45* might act as a negative regulator in response to biotic stresses. In this study, our analysis of *SbWRKY45* showed that the gene was up-regulated under the abiotic stress except under heat. This suggested that *SbWRKY45* gene had participated in drought, cold, and salt stress, possibly related to different *cis*-acting elements in the *SbWRKY45* promoter (Table 1). Transcriptome-based reports have proved that multiple pathways respond independently to abiotic stressors (Jiang et al., 2015). Our results showed that *SbWRKY45* exhibited drought tolerance.

5. Conclusion
We isolated and identified *SbWRKY45* gene from sorghum. *SbWRKY45* was differentially up-regulated by drought, cold, and salt treatments; however, it was weakly induced by heat. Overexpression of *SbWRKY45* improved drought tolerance in transgenic *Arabidopsis* plants. These results demonstrate that the *SbWRKY45* gene plays important roles in drought stress response; moreover, *SbWRKY45* enhanced seed germination and root development. *SbWRKY45* might be a potential candidate for molecular genetic modification of sorghum. These findings provide a basis for further functional analysis of sorghum *WRKYs* in response to abiotic stress.

Supplementary material
Supplementary material is available online at www.mdpi.com/xxx/s1, Table S1: *SbWRKY45* specific primers used in qRT-PCR analysis.

Author contributions
E.B. designed and performed the experiment and prepared the manuscript. P.X and Z.Z. reviewed the results and revised the manuscript. M.H. analyzed the results. All authors made substantial intellectual contributions to the work and approved it for publication. All authors read and agreed to the published version of the manuscript.

Funding

Conflicts of interest
The authors declare no conflicts of interest.

References

Ali MA, Azeem F, Nawaz MA, Acet T, Abbas A et al. (2018). Transcription factors WRKY11 and WRKY17 are involved in abiotic stress responses in Arabidopsis. Journal of Plant Physiology. 226: 12–21. doi: 10.1016/j.jplph.2018.04.007

Awlia M, Nigro A, Fajkus J, Schmoeckel SM, Negrão S et al. (2016). High-throughput non-destructive phenotyping of traits that contribute to salinity Tolerance in *Arabidopsis thaliana*. Frontiers in Plant Science (7): 1414. doi: 10.3389/fpls.2016.01414

Baillo EH, Hanif MS, Guo Y, Zhang Z, Xu P, et al. (2020). Genome-wide Identification of WRKY transcription factor family members in sorghum (*Sorghum bicolor* (L.) moench). PLoS ONE 15 (8): e0236651. doi: 10.1371/journal.pone.0236651

Baillo EH, Kimotho R, Zhang Z, Xu P (2019). Transcription factors associated with abiotic and biotic stress tolerance and their potential for crops improvement. Genes (10): 771. doi: 10.3390/genes10100771

Baloglu MC, Inal B, Kavas M, Unver T (2014). Diverse expression pattern of wheat transcription factors against abiotic stresses in wheat species. Gene 550 (1): 117-122. doi: 10.1016/j.gene.2014.08.025

Brotman Y, Landau U, Cuadros-Inostroza Á, TohgeT, Fernie AR et al. (2013). Trichoderma-plant root colonization: escaping early plant defense responses, and activation of the antioxidant machinery for saline stress tolerance. PLoS Pathogens 9 (4): e1003221. doi:10.1371/journal.ppat.1003221

Cai R, Zhao Y, Wang Y, Lin Y, Peng X et al. (2014). Overexpression of a maize WRKY58 gene enhances drought and salt tolerance in transgenic rice. Plant Cell Tissue Organ Culture 119: 565–577, doi: 10.1007/s11240-014-0556-7

Chen L, Song Y, Li S, Zhang L, Zou C et al. (2012). The role of WRKY transcription factors in plant abiotic stresses. Biochimica et Biophysica Acta 1819(2): 120–128. doi: 10.1016/j.bbagrm.2011.09.002

Chen X, Chen R, Wang Y, Wu C, Huang J et al. (2019b). Genome-wide identification of WRKY transcription factors in Chinese jujube (*Ziziphus jujuba* Mill.) and their involvement in fruit developing, ripening, and abiotic stress. Genes 10: 360. doi: 10.3390/genes10050360

Chen X, Li C, Wang H, Guo Z (2019a). WRKY transcription factors : evolution, binding, and action. Phytopathology Research (1): 1-15. doi: 10.1186/s42483-019-0022-x

Ciolkowski I, Wanke D, Birkenbihl RP, Somssich IE et al. (2008). Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. Plant Molecular Biology 68: 81–92. doi: 10.1007/s11103-008-9353-1

Dong X, Yang Y, Zhang Z, Xiao Z, Bai X et al. (2019). genome-wide identification of WRKY genes and their response to cold stress in *Coffea canephora*. Forests 10 (4): 335. doi: 10.3390/f10040335

El-Esawi MA, Al-Ghamdi AA, Ali HM, Ahmad M (2019). Overexpression of AtWRKY30 Transcription Factor Enhances Heat And Drought Stress Tolerance In Wheat (*Triticum aestivum* L.). Genes 10: 163. doi: 10.3390/genes10020163

Erpen L, Devi HS, Grosser JW, Dutt M (2018). Potential use of the DREB/ERF, MYB, NAC and WRKY transcription factors to improve abiotic and biotic stress in transgenic plants. Plant Cell Tissue Organ Culture 132:1–25. doi: 10.1007/s11240-017-1320-6

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000). The WRKY superfamily of plant transcription factors. Trends in Plant Science(5): 199–206. doi: 10.1016/S1360-1385(00)01600-9

Gao H, Wang Y, Xu P, Zhang Z (2018). Overexpression of a WRKY transcriptions factor TaWRKY2 enhances drought stress tolerance in transgenic wheat. Frontiers in Plant Science (9): 997. doi: 10.3389/fpls.2018.00997

He GH, Xu JY, Wang YX, Liu JM, Li PS et al. (2016). Drought-responsive WRKY transcription factor genes *TaWRKY1* and *TaWRKY33* from wheat confer drought and/or heat resistance in Arabidopsis. BMC Plant Biology (16): 1–16. doi: 10.1186/s12870-016-0806-4

Hu B, Jin J, Guo AY, Zhang H, Luo J et al. (2015). GSDS 2.0: An upgraded gene feature visualization server. Bioinformatics 31: 1296–1297. doi: 10.1093/bioinformatics/btu817

Jiang W, Wu J, Zhang Y, Yin L, Lu J et al. (2015). Isolation of a WRKY30 gene from *Muscadinia rotundifolia* (Michx) and validation of its function under biotic and abiotic stresses. Protoplasma 252: 1361–1374. doi: 10.1007/s00709-015-0769-6

Jiang Y, Liang G, Yu D (2012). Activated expression of WRKY57 confers drought tolerance in Arabidopsis. Molecular Plant 5 (6): 1375–1388. doi: 10.1093/mp/sss080

Jiao Z, Sun J, Wang C, Dong Y, Xiao S et al. (2018) Genome-wide characterization, evolutionary analysis of WRKY genes in Cucurbitaceae species and assessment of its roles in resisting to powdery mildew disease. PLoS ONE 13 (12): e0199851. https://doi.org/10.1371/journal.pone.0199851

Kim CY, Vo KT, Nguyen CD, Jeong DH, Lee SK et al. (2016). Functional analysis of a cold-responsive rice WRKY gene, OsWRKY71. Plant Biotechnology Reports (10): 13–23. doi: 10.1007/s11816-015-0383-2

Kimotho N, Baillo E, Zhang Z (2019). Transcription factors involved in abiotic stress responses in maize (*Zea mays* L.) and their roles in enhanced productivity in the post genomics era. PeerJ 8: e7211. doi: 10.7287/peerj.preprints.27549v1

Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis Version 7.0 for Bigger Datasets. Molecular Biologyand Evolution 33 (7): 1870–1874. doi: 10.1093/molbev/msw054

Lata C, Yadav A, Prasad M (2011). Role of plant transcription factors in abiotic stress tolerance. In: Shankar A and Venkateswarlu B (editors.), Abiotic StressResponse in Plants- Physiological, Biochemical and Genetic Perspectives. InTechOpen. doi: 10.5772/23172

Le DT, Nishiyama R, Watanabe Y, Mochida K, Yamaguchi-Shinozaki K et al. (2011). Genome-Wide expression profiling of soybean two-component system genes in soybean root and shoot tissues under dehydration stress. DNA Research (18): 17–29. doi: 10.1093/dnares/dsq032

Letunic I, Bork P (2018). 20 years of the SMART protein domain annotation resource. Nucleic Acids Research 46: 493–D496. doi:10.1093/nar/gkx922

Li H, Gao Y, Xu H, Dai Y, Deng D et al. (2013). ZmWRKY33, a WRKY maize transcription factor conferring enhanced salt stress tolerances in Arabidopsis. Plant Growth Regulation 70: 207–216. doi: 10.1007/s10725-013-9792-9

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 25 (4). 402–408. doi: 101006/meth.2001.1262

Ma B, Yin CC, He SJ, Lu X, Zhang WK et al. (2014). Ethylene-induced inhibition of root growth requires abscisic acid function in rice (*Oryza sativa* L.) seedlings. PLoS Genetics 10. doi: 10.1371/journal.pgen.1004701

Niu CF, Wei W, Zhou QY, Tian AG, Hao YJ et al. (2012). Wheat WRKY genes *TaWRKY2 a*nd *TaWRKY19* regulate abiotic stress tolerance in transgenic Arabidopsis plants. Plant Cell Environment 35 (6): 1156–1170. doi: 10.1111/j.1365-3040.2012.02480.x

Osmolovskaya N, Shumilina J, Kim A, DiDio A, Grishina T et al. (2018). Methodology of drought stress research: experimental setup and physiological characterization. International Journal of Molecular Sciences 19: 4089. doi: 10.3390/ijms19124089

Schön M, Toller A, Diezel C, Roth C, Westphal L et al. (2013). Analyses of wrky18 wrky40 plants reveal critical roles of SA/EDS1 signaling and indole-glucosinolate biosynthesis for *Golovinomyces orontii* resistance and a loss-of resistance towards *Pseudomonas syringae* pv. tomato AvrRPS4. Molecular Plant Microbe Interaction 26: 758–767. doi:10.1094/MPMI-11-12-0265-R

Tuleski TR, Kimball J, do Amaral FP, Pereira TP, Tadra-Sfeir MZ et al. (2020). *Herbaspirillum rubrisubalbicans* as a phytopathogenic model to study the immune system of *Sorghum bicolor*. Molecular Plant Microbe Interaction 33(2): 235-246. doi: 10.1094/MPMI-06-19-0154-R

Varoquaux N, Cole B, Gao C, Pierroz G, Baker CR et al. (2019). Transcriptomic analysis of field-droughted sorghum from seedling to maturity reveals biotic and metabolic responses. PNAS 146: 1983-1995. doi: 10.1073/pnas.1907500116

Wang CT, Ru JN, Liu YW, Li M, Zhao D et al. (2018a). Maize WRKY transcription factor ZmWRKY106 confers drought and heat tolerance in transgenic plants. International Journal of Molecular Sciences 19: 1–15, doi: 10.3390/ijms19103046

Wang CT, Ru JN, Liu YW, Yang JF, Li M et al. (2018b). The Maize WRKY transcription factor ZmWRKY40 confers drought resistance in transgenic Arabidopsis. International Journal of Molecular Sciences 19: 2580. doi: 10.3390/ijms19092580

Wang NN, Xu SW, Sun YL, Liu D, Zhou L et al. (2019). The cotton WRKY transcription factor (*GhWRKY33*) reduces transgenic Arabidopsis resistance to drought stress. Scientific Reports (9): 1–13. doi: 10.1038/s41598-018-37035-2

Wang P, Yue C, Chen, D, Zheng Y, Zhang Q et al. (2018). Genome-wide identification of WRKY family genes and their response to abiotic stresses in tea plant (*Camellia sinensis*). Genes and Genomics 41: 17–33. doi: 10.1007/s13258-018-0734-9

Waqas M, Azhar MT, Rana IA, Azeem F, Ali MA et al. (2019). Genome-wide identification and expression analyses of WRKY transcription factor family members from chickpea (*Cicer arietinum* L.) reveal their role in abiotic stress-responses. Genes and Genomics 41: 467–481. doi: 10.1007/s13258-018-00780-9

Zhang L, Zhao T, Sun X, Wang Y, Du C et al. (2019). Overexpression of *VaWRKY12*, a transcription factor from *Vitis amurensis* with increased nuclear localization under low temperature, enhances cold tolerance of plants. Plant Molecular Biolology 100: 95–110. doi: 10.1007/s11103-019-00846-6

Zhang Y, Yu H, Yang X, Li Q,, Ling J et al. (2016). CsWRKY46, a WRKY transcription factor from cucumber, confers cold resistance in transgenic-plant by regulating a set of cold-stress responsive genes in an ABA-dependent manner. Plant Physiology and Biochemistry 108: 478–487. doi: 10.1016/j.plaphy.2016.08.013

Zhao K, Zhang D, Lv K, Zhang X, Cheng Z et al. (2019). Functional characterization of poplar WRKY75 in salt and osmotic tolerance. Plant Science 289: 110259. doi: 10.1016/j.plantsci.2019.110259