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Genome-wide identification and expression analysis of the *WRKY* genes in sugar beet (*Beta vulgaris* L.) under alkaline stress

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Background: The WRKY transcription factor family plays critical roles in many aspects of physiological processes and adaption to environment. Although the *WRKY* genes have been widely studied in various plants, the structure and function of the *WRKY* family in sugar beet (*Beta vulgaris* L.) remains unknown.

Methods: In the present study, the *WRKY* genes were identified from the sugar beet genome by bioinformatics. Phylogenetic tree was constructed by MEGA7.0 software. Distribution map of these genes was displayed by MapInspect 1.0. Furthermore, the exon-intron structure and the conserved motifs were predicted by GSDS 2.0 and MEME 5.0.5, respectively. Additionally, the expression levels of these genes under alkaline stress were assayed by qRT-PCR.

Results: A total of 58 putative *BvWRKY* genes are identified in the sugar beet genome. The coding sequence of these genes ranged from 558 to 2,307 bp and molecular weight varied from 21.3 to 84. Based on the conserved WRKY domain and zinc-finger motif, the *BvWRKY* genes are clustered into three major groups I, II and III, with 11, 40 and 7 genes, respectively. The number of intron in the *BvWRKY* genes ranged from 1 to 5, with majority of *BvWRKY* (27/58) containing three exons. All the *BvWRKY* genes have one or two WRKY motifs at the N-terminus. Moreover, the expression levels of *BvWRKY* genes are increased remarkedly by alkaline stress. Our findings extend understandings of the *BvWRKY* genes family and provide useful information for subsequent research on their functions in sugar beet under alkaline stress.

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ABSTRACT

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Results: A total of 58 putative *BvWRKY* genes are identified in the sugar beet genome. The coding sequence of these genes ranged from 558 to 2,307 bp and molecular weight varied from 21.3 to 84. Based on the conserved WRKY domain and zinc-finger motif, the *BvWRKY* genes are clustered into three major groups I, II and III, with 11, 40 and 7 genes, respectively. The number of intron in the *BvWRKY* genes ranged from 1 to 5, with majority of *BvWRKY* (27/58) containing three exons. All the *BvWRKY* genes have one or two WRKY motifs at the N-terminus. Moreover, the expression levels of *BvWRKY* genes are increased remarkedly by alkaline stress. Our findings extend understandings of the *BvWRKY* genes family and provide useful information for subsequent research on their functions in sugar beet under alkaline stress.

Keywords: Sugar beet, WRKY transcription factor, Bioinformatics, Alkaline stress, Expression levels

INTRODUCTION

Plants encounter various abiotic and biotic stresses throughout their life cycle. These stresses can adversely affect the growth and development of plants and/or change the distribution of species (Prachi et al., 2017). To cope with these adversely environments, plants have developed a series of mechanisms at the morphological, physiological and molecular levels during the process of long-term evolution (Hasanuzzaman et al., 2013; Bechtold et al., 2017; Yang & Guo, 2018). So far, the responses of abiotic stress and the regulation of genes have been widely reported in various plant species, such as *Arabidopsis thaliana* (Kotchoni et al., 2006), rice (*Oryza sativa*) (Lee et al., 2005), tomato (*Lycopersicon esculentum*) (Sharma et al., 2010), and wheat (*Triticum aestivum*) (Zhang et al., 2011). It is well-documented that several families of genes are particularly related to obvious improvement in abiotic stress tolerance of plants, including the *WRKY*, *NAC*, *MYB*, and *GRF* genes families (Chinnusamy, Zhu & Zhu, 2006; Hennig, 2012; Cao et al., 2017; Khadiza et al., 2017).

The WRKY family, one of the most important transcription factors (TFs) in plants, plays critical roles in various aspects of physiological processes as well as response to biotic and abiotic stresses (Jiang et al., 2016). Ishiguro & Nakamura (1994) identified the first *WRKY* gene *SPF1* from sweetpotato (*Ipomoea batatas*). After that, a large proportion of *WRKY* genes have been characterized in different kind of plants, especially some grass species, such as barley (*Hordeum vulgare*) (Mangelsen et al., 2008), *Brachypodium distachyon* (Wen et al., 2014), maize (*Zea mays*) (Wei et al., 2016), rice (Ross, Liu & Shen, 2010), and wheat (Zhu et al., 2013). All the members of the *WRKY* genes have been documented to have one or two conserved WRKY domains, which are composed of approximately 60 amino acids with "WRKYGQK" at N-terminus and a zinc-finger motif C-X_{4-5}-C-X_{22-23}-H-X-H (C_2H_2) or C-X_7-C-X_{23}-H-X-C (C_2HC) at C-terminus (Eulgem et al., 2000; Rushton et al., 2010). Based on the number and characteristics of the conserved WRKY domains, the *WRKY* genes were divided into three groups I, II, and III (Eulgem et al., 2000). There are evidences that members of group I *WRKY* displayed two WRKY domains with zinc-finger motifs of C_2H_2, group II contained only single WRKY domain with a zinc-finger motif of C_2H_2, whereas group III had one WRKY domain with a zinc-finger motif of C_2HC (Rushton et al., 2010). Additionally, group II *WRKYs* can be further classified into five distinct subgroups IIa, IIb, IIc, IId, and IIe (Rushton et al., 2010; Ulker & Somssich, 2004). It was reported that the *WRKY* genes were induced significantly by various

environmental factors, such as heat (Li et al., 2009), drought (Jiang, Gang & Yu, 2012), waterlogging (Li et al., 2017), cold (Luo et al., 2017), and salt stress (Dan et al., 2018), indicating that the *WRKY* genes play the positive regulatory functions in plants when exposed to adversely stressed conditions. In *Arabidopsis*, overexpression of *TaWRKY1* and *TaWRKY33* has been shown to activate the expression of several downstream genes related to stress response, increase the germination rate of seeds, and promote the growth of roots in transgenic plants subjected to various stresses (He et al., 2016). Compared to wild-type (WT) lines, *TaWRKY33* overexpressing *Arabidopsis* lines displayed a relatively lower water-loss rate during dehydration stress (He et al., 2016). In P-deficient conditions, *OsWRKY74* overexpressing rice exhibited greater accumulation of iron (Fe) and up-regulation of the cold-responsive genes compared with WT plants (Dai, Wang & Zhang, 2016). Under saline condition, the activities of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in *Chrysanthemum* overexpressed *DgWRKY5* gene were obviously higher than those of WT plants, whilst the accumulation of H_2O_2, O^{2-}, and malondialdehyde (MDA) were significantly lower than those of WT plants (Liang et al., 2017). Moreover, the expression levels of genes related stress such as *DgCAT*, *DgAPX*, *DgNCED3A*, *DgCuZnSOD*, *DgNCED3B*, *DgCSD1*, *DgCSD2*, and *DgP5CS* were remarkably higher in transgenic *Chrysanthemum* plants than those in WT plants, indicating that DgWRKY5 might be a positive regulatory factor in response to salt stress (Liang et al., 2017). These results documented that the *WRKY* genes may provide valuable insights into abiotic stress tolerance mechanisms in plants.

Sugar beet ($2n = 18$, *Beta vulgaris* L.), belonging to the order of Caryophyalles, is a major sugar crop worldwide, which provides approximately 30% of the world's sugar production (Liu et al., 2010). In China, this crop is cultivated in the arid and semi-arid regions of Northern China (Wu et al., 2013). The whole-genome sequence of sugar beet was completed and released in 2014 and a total of 359.14 Mb of sequence data was assembled with 27,421 protein-coding genes predicted (Dohm et al., 2014). Our previous studies indicated that the addition of 50 mM NaCl in the growth medium can stimulate the growth of plants and mitigate the damage caused by osmotic stress in sugar beet (Wu et al., 2015). Recently, our results showed that 15–25 mM $NaHCO_3$ have no effects on the growth of plants, whereas higher concentrations (50–100 mM) of $NaHCO_3$ significantly reduced both fresh and dry weights of shoots and roots in sugar beet (Wu et al., unpublished data). Furthermore, Na^+ concentrations in roots and shoots displayed a

sharply increased trend with the increase of $NaCHO_3$ concentrations, whereas K^+ concentrations maintained a relatively stable level under either low- or high- alkaline condition. These results implied that the maintenance of K^+ and Na^+ homeostasis might be an important strategy for sugar beet adapting to alkaline stress. However, the WRKY family genes and their regulated expression under alkaline stress in sugar beet remain unknown.

Here, we proposed a hypothesis that the *WRKY* genes play a positive regulatory function in response to alkaline stress in sugar beet. To test this hypothesis, in this study, firstly, a total of 58 *BvWRKY* genes were identified in the sugar beet genome, and the phylogenetic relationship, chromosome distribution, genes structure and conserved motifs were analyzed; Secondly, the expression patterns of the *BvWRKY* genes in roots and shoots of sugar beet seedlings exposed to different concentrations (15–100 mM) of $NaCHO_3$ were determined by qRT-PCR. Our findings extend understandings of the *BvWRKY* family genes and provide useful information for subsequent research on their functions in sugar beet under alkaline stress.

MATERIALS AND METHODS

Identification and distribution of the *WRKY* genes in sugar beet

To identify the sugar beet *WRKY* family genes, protein sequences of the *WRKY* genes in *Arabidopsis* were downloaded from The *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org/), and used queries in performing on Basic Local Alignment Search Tool of Protein (BLASTP) searches with default algorithm parameters on The *Beta vulgaris* Resource (bvseq.boku.ac.at/) (Dohm et al., 2014) and NCBI sugar beet genome data (https://www.ncbi.nlm.nih.gov/nuccore/?term=Beta+vulgaris+subsp.+vulgaris). The local Hidden Markov Model-based (HMM) search program (https://www.ebi.ac.uk/Tools/hmmer/) was used to identify *WRKY* in sugar beet and *Arabidopsis* (Potter et al., 2018).

The isoelectric points (pIs) and theoretical molecular weights (MWs) of each BvWRKY protein were predicted online at https://web.expasy.org/protparam/ (Gasteiger et al., 2005). Based on the position information from the sugar beet genome database, the *BvWRKY* genes were plotted on the chromosomes and the distribution map of *BvWRKY* genes was displayed by MapInspect 1.0 (https://mapinspect.software.informer.com/).

Phylogenetic analysis, gene structure and conserved motifs distribution

The amino acid sequences of *BvWRKY* genes were aligned with those of *AtWRKYs* from

Arabidopsis (Table S1), by using the Clustal W version 2.0 (http://www.clustal.org/clustal2/) (Larkin et al., 2007). Phylogenetic tree was built by MEGA7.0 (https://www.megasoftware.net/) using the neighbor-joining (NJ) algorithm with 1,000 bootstraps. The exon-intron structure of the *BvWRKY* genes were predicated via Gene Structure Display Server (GSDS version 2.0, http://gsds.cbi.pku.edu.cn/) according to the alignment of their coding sequences (CDS) with their corresponding genomic sequences (Hu et al., 2015). were analyzed by Multiple Em for Motif Elicitation (MEME version 5.0.5, http://meme-suite.org/tools/meme) was used to predict the conserved motifs of the *BvWRKY* genes (Bailey & Elkan, 1994), and the parameters used in this study were set as follows: maximum number of different motifs is 10, other default parameters.

Plant materials, treatments and qRT-PCR analysis

Seeds of sugar beet (*B. vulgaris* L.) cultivar Gantang7 were kindly provided by Mr. Shengfu Duan from Wuwei Sannong Seed Technology Co., Ltd., Gansu, China, in May 2018. Seeds were surface sterilized for 1 min in 75% ethanol (*v/v*) and rinsed three times with sterilized distilled water, soaked in sterilized water for 24 h (Wu et al., 2013; 2015), and then planted in plastic containers filled with vermiculite and watered with the modified Hoagland nutrient solution including 2 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $Ca(NO_3)_2$, 18 mM $MnCl_2{\cdot}4H_2O$, 1.6 mM $ZnSO_4{\cdot}7H_2O$, 0.6 mM $CuSO_4{\cdot}5H_2O$, 0.5 mM $MgSO_4$, 60 mM Fe-Citrate, 92 mM H_3BO_3, and 0.7 mM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. All the seedlings were grown in the growth room with the temperature of 25/20 °C (day/night), the daily photoperiod of 16/8 h (day/night), the relative humidity of 65–75%, and the light density of 550–600 $mmol{\cdot}m^{-2}{\cdot}s^{-1}$ during the photoperiod.

Four-week-old seedlings were treated with modified Hoagland nutrient solution supplemented with additional 0 (Control), 15, 25, 50, and 100 mM $NaHCO_3$. Shoot and root tissues were collected at 72 h after $NaHCO_3$ treatments, respectively. Samples of shoots and roots were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

The total RNA was isolated from roots or shoots of sugar beet using UNlQ-10 Column Trizol Total RNA Isolation kit (Sangon, Shanghai, China) according to the manufacturer's procedure. The first strand of complementary DNA was synthesized using a PrimeScript™ Real-Time (RT) Master Mix kit (Takara, Dalian, China) according to the manufacturer's instruction. qRT-PCR with a TB Green™ Premix Ex Taq™ II kit (Takara, Dalian, China) was performed using a MA-6000 RT-PCR System (Molarray, Suzhou, China). The qRT-PCR reaction

conditions were as following: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 1 min. The expression level of each gene was determined according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), and all analyses included at least three biological replicates. The *BvACTIN* gene was used as the internal control. All primers of the *BvWRKY* genes used for are qRT-PCR are listed in Table 1.

RESULTS

Identification and characterization of the *BvWRKY* genes

A total of 64 transcripts in the sugar beet genome sequences is identified as candidate members of the *WRKY* genes. After removing the overlapping sequences manually, a total of 58 sequences are eventually identified as *WRKY* genes and named from *BvWRKY1* to *BvWRKY58* according to their order in the sugar beet genomic sequence (Table 2). The sequence analysis of the *BvWRKY* genes showed that CDS ranges from 558 (*BvWRKY46*) to 2,307 bp (*BvWRKY7*) and predicted proteins ranged from 185 to 768 amino acids (aa) in length with an average of approximately 387 aa. The MWs varied from 21.3 (*BvWRKY46*) to 84.2 kDa (*BvWRKY7*). The pIs ranged from 5.4 (*BvWRKY34*) to 9.9 (*BvWRKY28*), with 22 pIs >7 and the remaining pIs ≤7 (Table 2). Similar observations were made in sesame (*Sesamum indicum*) (Li et al., 2017), which has 71 *SiWRKY* with MWs ranging from 14.4 to 125.9 and pIs ranging from 4.8 to 9.7.

Phylogenetic analysis of the *BvWRKY* genes

To explore the phylogenetic and evolutionary relationship of the *WRKY* genes in sugar beet and group them with the established subfamilies, we investigated 118 amino acid sequences containing the conserved WRKY domain. These sequences consisted of 58 sequences from sugar beet and 60 sequences from *Arabidopsis* (Table S1). An unrooted neighbor-joining (NJ) phylogenetic tree was built according to multiple alignments of the predicted amino acid sequences by MEGA 7.0 software. In the phylogenetic tree, the *BvWRKYs* genes are classified into three major groups: *BvWRKY* I, II, and III, based on their putative WRKY domains and zinc-finger motifs. There are 11 members in *BvWRKY* group I, 40 members in group II, and 7 members in group III. Furthermore, group II is divided into five subgroups: *BvWRKY* IIa, b, c, d, and e, containing 3, 7, 15, 7, and 8 genes, respectively (Fig. 1 and Table 2).

Distribution of the *BvWRKY* genes on the sugar beet chromosomes

Out of 58, 55 of *BvWRKY* genes were displayed on the sugar beet chromosomes. As shown

in Fig. 2, 55 *BvWRKY* genes are evenly distributed throughout all nine sugar beet chromosomes, and the number on each chromosome is not necessarily correlated with its length. Chr2, 5, and 6 had relatively more *BvWRKY* genes, with 9, 11, and 9 genes, respectively. Chr3 and 7 contained relatively fewer *BvWRKY* genes, with only 3 genes, respectively. Eleven out of 58 (18.9%) *BvWRKY* genes are located on Chr5, while the sequenced size of Chr5 (50.03 Mb) only accounts for about 13.96% of the assembled sugar beet genome (359.14 Mb), indicating that the *BvWRKY* genes are enriched in Chr5. Unfortunately, 3 genes, viz *BvWRKY40, -41*, and *-42*, are putatively located on the "Chromosomes Unknown" (Table 2).

Two or more homologous genes within a 100 Kb range distance were defined as tandem duplicates. Nine tandem duplication regions clustered with 19 *BvWRKY* genes, including 3 genes in group I, 2 genes in group IIa, 2 genes in group IIb, 6 genes in group IIc, 2 genes in group IId, and 4 genes in group IIe, are identified on Chr2 (*BvWRKY1, -2, -8* and *BvWRKY33, -34*), Chr5 (*BvWRKY5, -54*), Chr6 (*BvWRKY20, -21* and *BvWRKY18, -53*), Chr8 (*BvWRKY14, -15* and *BvWRKY43, -44*), and Chr9 (*BvWRKY11, -12* and *BvWRKY13, -50*), respectively (Fig. 2 and Table 2).

Conserved motifs and structure of the *BvWRKY* genes

To further investigate the structural characteristics of the *BvWRKY* genes, the conserved motifs and intron/exon distribution were analyzed according to their phylogenetic relationships. A total of 10 putatively conserved motifs are observed in the *BvWRKY* genes using the MEME 5.0.5 software and further annotated by InterPro Scan 5 (Jones et al., 2014). It was showed that four (motif 1, -2, -3, and -6) of 10 motifs are annotated as WRKY DNA-binding, which is a basic feature of the *WRKY* genes families. All the *BvWRKY* genes contained motif 1 and -2, indicating that the sugar beet *WRKYs* identified in this study have conserved features of the *WRKY* family. Notably, eleven genes (*BvWRKY1, -2, -3, -4, -6, -7, -8, -9, -10, -51,* and *-58*) also contain motif 3 and -6 (Fig. 3), indicating that these genes have two conserved WKRY domains, which are consistent with results of the phylogenetic tree (Fig. 1). Interestingly, motif 5, -7, -8, and -9 are unique within group II, whereas motif 10 is unique within group I (Fig. 3). Generally, the *WRKY* genes in the same cluster commonly share similar motif compositions, suggesting functional similarity among them.

To determine the structural diversity of the *BvWRKY* genes, the distribution of intron-exon was analyzed and compared. The number of introns in the *BvWRKY* genes ranges from 1

(*BvWRKY36, -45, -46*, *-47*, *-52*, and *-55*) to 5 (*BvWRKY1, -2*, *-6*, *-7, -8*, *-10*, *-14, -15*, *-41*, and *-42*). Thus, sequence of each *BvWRKY* gene was divided into many segments by introns and the average number of exons among the full-length *WRKY* genes in the common sugar beet genome was 3.52. It is found that 31 of 58 *BvWRKY* genes contain the typical splicing of three exons and two introns (Fig. 4). These results suggested that *BvWRKY* genes families within the same group generally share a similar structure.

Expression analysis of the selected *BvWRKY* genes in response to alkaline stress

To provide some clues on the roles of *BvWRKY* genes in response to alkaline stress, nine genes, namely *BvWRKY1*, *-4*, *-6*, *-9*, *-17*, *-31*, *-36, -44*, and *-55*, were selected to determine their expression levels by qRT-PCR. The results showed that the transcript abundances of the *BvWRKY* genes are remarkedly altered by alkaline stress (Fig. 5). Of these, five *BvWRKY* genes, namely *BvWRKY1*, *-4*, *-9*, *-17*, and *-31* are induced significantly in both shoot and root tissues at different concentrations following the start of $NaHCO_3$ treatment. Importantly, with the increase of $NaHCO_3$ concentrations, the expression of *BvWRKY1* in shoots displayed a significant up-regulation, and then reached a peak value at 50 mM $NaHCO_3$ which is 2.2-fold higher than that at control condition (Fig. 5a). The transcript abundances of *BvWRKY17*, *-31*, and *-44* are increased significantly in roots; the peak values of *BvWRKY17* and *-31* occur when concentration of $NaHCO_3$ is 50 mM, while the expression peak value of *BvWRKY44* appear when concentration of alkaline is 25 mM (Fig. 5h). Interestingly, the expression level of *BvWRKY31* in roots is up-regulated by 13-fold at 50 mM $NaHCO_3$ compared to control (Fig. 5f).

DISCUSSION

The *WRKY* family has been widely identified in various organisms, including spike mosses, single-celled green algae, slime molds and protozoa (Rushton et al., 2010). In monocots and dicots, including soybean (*Glycine max*) (Luo et al., 2013), wheat (Qin, Tian & Liu, 2015), rice (Dai, Wang & Zhang, 2016), and cotton (*Gossypium hirsutum*) (Liu et al., 2016), an especially large number of *WRKY* genes have been documented to have various functions in recent years. In the present study, the *WRKY* genes were firstly identified from whole-genome sequences of sugar beet.

To date, whole genomes of many plants have been sequenced and a large of the *WRKY* genes have been identified in different plant species (Wu, 2005; Wei et al., 2012; Dou et al.,

2014; Yu et al., 2016; Yue et al., 2016; Jing et al., 2017). Completion of the sugar beet genome makes it possible to analyze the *WRKY* genes at the whole genome level (Dohm et al., 2014). In the present study, a total of 58 putative *BvWRKY* genes are identified in the sugar beet genome (Table 2). It was observed that there are 32 *WRKY* genes in broomcorn millet (*Panicum miliaceum*) (Yue et al., 2016), 71 in sesame (Li et al., 2017), 85 in cassava (*Manihot esculenta*) (Wei et al., 2016), 88 in common bean (*Phaseolus vulgaris*) (Jing et al., 2016), 100 in rice (Wu, 2005), 103 in *Aegilops tauschii* (Ma et al., 2014), 116 in *Gossypium raimondii* (Dou et al., 2014), and 136 in maize (Wei et al., 2012). These findings showed that there are large differences in number of the *WRKY* genes families among different plant species.

Gene replication events play critical roles in rapid expansion and the evolution of genes families (Cannon et al., 2004). It is well-known that genes within a single genome are divided into five distinct classes: singletons, dispersed-, proximal-, tandem- and segmental/whole genome duplication (WGD)-duplicates, respectively, according to the copy number of genes and the distribution of genome (Wang et al., 2012). It was documented that duplication events can lead to a clustered occurrence of family members via tandem amplification, or a scattered occurrence via segmental duplication of chromosomal regions (Grassi, Lanave & Saccone, 2008). In the present study, it was found that 32.8% (19/58) of the *BvWRKY* genes evolve from tandem repeats (Fig. 2). Tandem gene replication of *WRKY* has been found in *Arabidopsis* (Cannon et al., 2004), rice (Ross, Liu & Shen, 2010), cucumber (*Cucumis sativus*) (Ling et al., 2011), and soybean (Yu et al., 2016). Therefore, we proposed that tandem gene replication might play important roles in the expansion of the *BvWRKY* family genes in sugar beet.

These are consistent with the classification of the *WRKY* family genes in *Arabidopsis* (Eulgem et al., 2000), maize (Wei et al., 2012), *Populus* (Jiang et al., 2014), cassava (Wei et al., 2016), and peach (*Prunus persica*) (Chen et al., 2016). All the *WRKY* genes can be classified into three distinct clusters: group I, II, and III depending on the number of conserved WRKY regions and the pattern of zinc-finger motif (Eulgem et al., 2000; Wei et al., 2012; Jiang et al., 2014). There are evidences that genes of group I included double conserved WRKY domains, which can interact with the W-box "TTGACY" core motif to activate downstream genes, and C_2H_2 zinc-finger motif; group II only possessed single WRKY domain and shared the same zinc-finer motif as group I; whereas group III had one conserved WRKY domain and C_2HC zinc-finger motif (Eulgem et al., 2000; Rushton et al., 2010). In our study, 11, 40, and 7 *BvWRKY* genes

have been classified into groups I, II, and III, respectively (Fig. 1 and Table 1). Previous studies have documented that group III was the largest group of *WRKY* genes families in rice and broomcorn millet, which accounted for 38% and 50% (Ross, Liu & Shen, 2010; Yue et al., 2016), while in *Arabidopsis* and sesame, group II was the largest group, accounting for 24% and 68% (Eulgem et al., 2000; Li et al., 2017), respectively. In the present study, group II has been also found to be the largest group of *WRKY* genes family in sugar beet, accounting for 69% of all the *BvWRKY* genes, which are consistent with the results of *Arabidopsis* and sesame but different from rice and broomcorn millet. Furthermore, group II can be divided into five distinct subgroups IIa, b, c, d, and e, according to the amino acid sequences outside the WRKY domain (Table 2 and Fig. 1). Subgroup IIc is also found to be the largest subgroup, accounting for 37.5% of all the genes of group II (Table 2), which is in accordance with the results reported in soybean (Luo et al., 2013), *Arabidopsis* (Qin et al., 2015), rice (Dai et al., 2016), and cotton (Liu et al., 2016). Furthermore, there are closely evolutionary relationships between group IIa and IIb, and between group IId and IIe (Fig. 1), respectively, which appear to make up monophyletic clades. Additionally, according to the distance of phylogenetic relationship, three groups of the *BvWRKY* genes can be clustered in four major lineages: group IIc + I, group IId + IIe, group III, and group IIa + IIb, respectively (Fig. 1). Similar lineages were also found in the *MdWRKY* genes families from apple (Lui et al., 2017). These results further confirmed that the WRKY family genes are highly conserved family in different plant species.

The number of motifs in *BvWRKYs* ranges from 2 to 6, and the length of motifs varies from 15 to 50 amino acids (Fig. 3 and Fig. S1). In addition, 4 motifs, namely motif 1, -2, -3, and -6 are found in the WRKY DNA-binding domain. Similar motifs were reported in *SiWRKYs* from sesame (Li et al., 2017). The other 6 motifs are found to be located outside in the WRKY domain. It is clear that motif 1 and -2 are shared by all the *BvWRKY* genes, while motif 3 and -6 were shared by 11 genes, viz *BvWRKY1*, *-2*, *-3*, *-4*, *-6*, *-7*, *-8*, *-9*, *-10*, *-51*, and *-58* (Fig. 3), which belonged to members of group I (Table 2). What is more important, motif 5, -7, -8, and -9 were shared by members of group II, and motif 10 was shared by group I. It is clear that members of *WRKY* in the same cluster commonly shared similar motif compositions, indicating functional similarity among them in sugar beet.

The structural diversity of exon/intron, an important part in the evolution of genes families, provides additional evidences supporting phylogenetic classification (Bleecker, 2003; Wang et

al., 2014). In the present study, the number of introns found in the *BvWRKY* genes ranges from 1 to 5, with an average of 2.79 introns per *BvWRKY*, so each *BvWRKY* sequence was divided into many segments by introns. Similarly, all of the *WRKY* genes in both cassava and peach have one to five introns (Wei et al., 2006; Chen et al., 2016). However, the *SiWRKY* genes in sesame have between 1 to 11 introns (Li et al., 2017). These results implied the diversity of the *WRKY* gene structures in various species. Moreover, the largest fraction of *BvWRKYs* (27, 46.6%) have two introns (Fig. 4), which is common in other plants, including cassava (42 of 85) (Wei et al. 2006), peach (29 of 58) (Chen et al., 2016), and sesame (33 of 71) (Li et al., 2017).

As one of the most important TFs in plants, the *WRKY* family has been found to play a pivotal role under abiotic stresses, especially salt stress (Zhou et al., 2015; Liang et al., 2017; Lui et al., 2017; Wang et al., 2017; Wu et al., 2017). In cucumber and tomato, the majority of *WRKYs* were remarkedly up-regulated only by salinity (Ling, Wang & Jiang, 2011; Huang et al. 2012). In *B. distachyon*, however, the most *BdWRKY* genes were significantly down-regulated by various abiotic stresses (Wen et al., 2014). In our study, nine *BvWRKY* genes, namely *BvWRKY1*, *-4*, *-6*, *-9*, *-7*, *-31*, *-36*, *-44*, and *-55* were responsive to alkaline stress in sugar beet (Fig. 5). Interestingly, *BvWRKY1* in shoots and *BvWRKY31* in roots are induced significantly and up-regulated rapidly (Fig. 5a, f), respectively, when plants are exposed to alkaline stress. What is more important, the mRNA levels of *BvWRKY31* in roots at 50 mM $NaHCO_3$ were 12-fold higher than those under control condition (0 mM $NaHCO_3$) (Fig. 5f). As early as 2010, it was reported that the key role of *Puccinellia tenuiflora* in response to alkaline stress was the accumulation of rhizospheric organic acids (Guo, Shi & Wang, 2010). We hypothesized *BvWRKY31* may be involved in regulating the expression of organic acid genes in response to the effects of high pH under alkaline stress needs to be further addressed. Overall, our results provide useful information for studying the effects of the *BvWRKY* genes in sugar beet under alkaline stress.

CONCLUSION

In the present study, a total of 58 putative *BvWRKY* genes were identified in the sugar beet genome. Based on the conserved WRKY domain and zinc-finger motif, the *BvWRKY* genes are classed into three major groups I, II, and III, each with 11, 40, and 7 genes, respectively. Of these, group II can be divided into five distinct subgroups IIa, b, c, d, and e. It was showed that all of the identified *BvWRKY* genes have the highly conserved WRKY domain. The number of

introns in the *BvWRKY* genes range from 1 to 5, with the majority of *BvWRKYs* containing 3 exons. Furthermore, all the detected *BvWRKY* genes are induced significantly by alkaline stress. It is clear that transcript levels of *BvWRKY1* in shoots and *BvWRKY31* in roots are significantly higher than those of other genes when plants are exposed to alkaline stress. This study provides a wide identification of the *BvWRKY* genes, and would be helpful for the improvement of alkaline tolerance in sugar beet by genetic engineering.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Guo-Qiang Wu conceived and designed the experiments, prepared the figures and/or tables, drafted the work or revised it critically for important content, approved the final draft.
- Zhi-Qiang Li performed the experiments, analyzed the data, prepared the figures and/or tables, approved the final draft.
- Han Cao and Jin-Long Wang performed the experiments, approved the final draft.

Supplementary Information

Figure S1 Detailed information of BvWRKY motifs in sugar beet.

Table S1 Sequences of the *WRKY* genes from sugar beet and *Arabidopsis*.

Table S2 The expression data of *BvWRKY* genes.

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Figure 1

Phylogenetic tree of *WRKY*genes in sugar beet (*Beta vulgaris*, *Bv*) and *Arabidopsis thaliana* (*At*).

The predicted proteins sequences of 58 *BvWRKYs* and 60 *AtWRKYs* were aligned by the Clustal W software and the phylogenetic tree was constructed using the MEGA7.0 software by the NJ method with 1,000 bootstrap replicates. *WRKY* genes were clustered into three major groups. Details of *BvWRKYs* and *AtWRKYs* were listed in Table S1.

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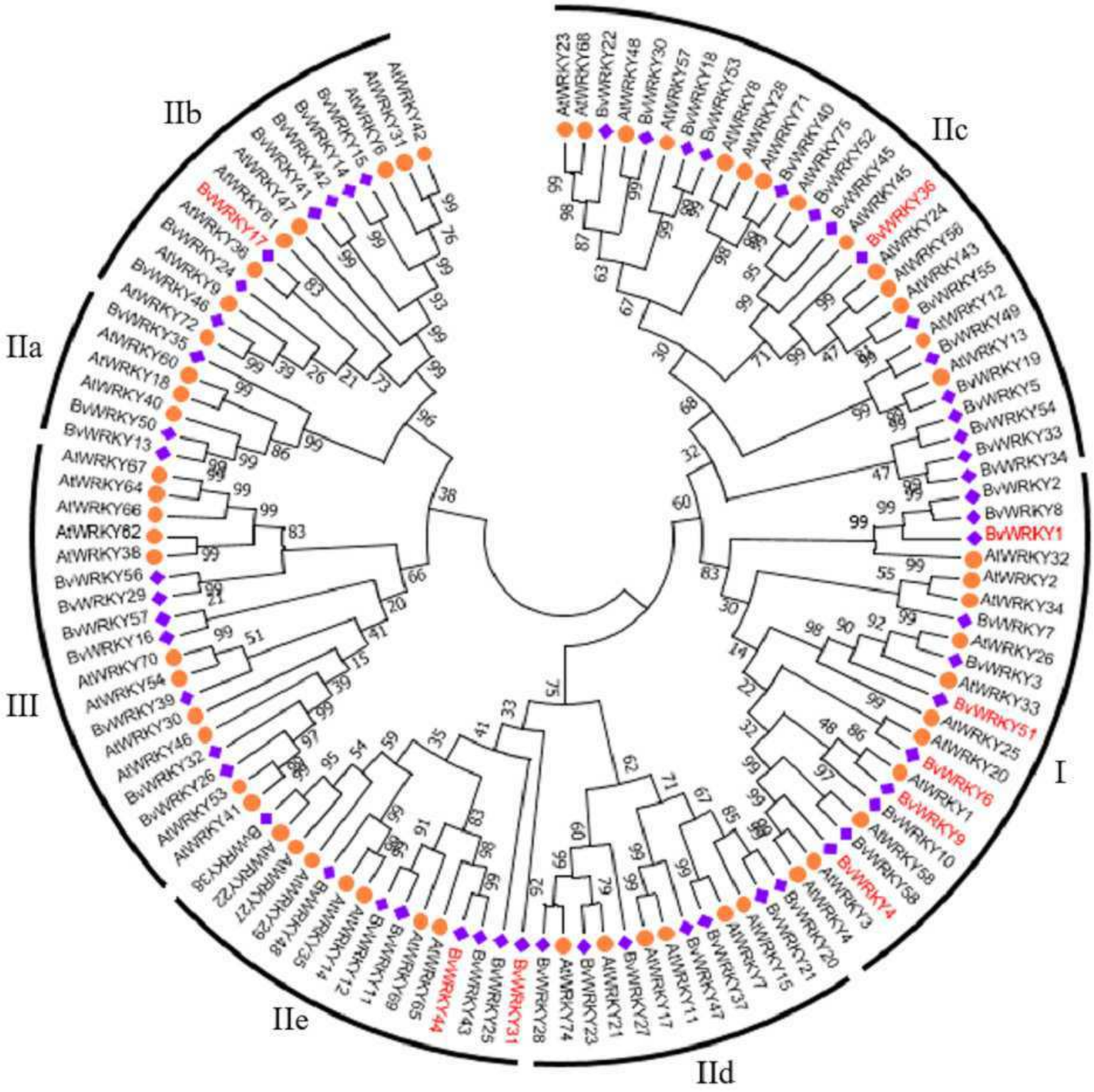

Figure 2

Distribution of *BvWRKY* geneson the nine sugar beet chromosomes.

The chromosome number was indicated at the top of each chromosome. The scale of the genome size was given on the left. The selected *BvWRKY* genes are indicated in red.

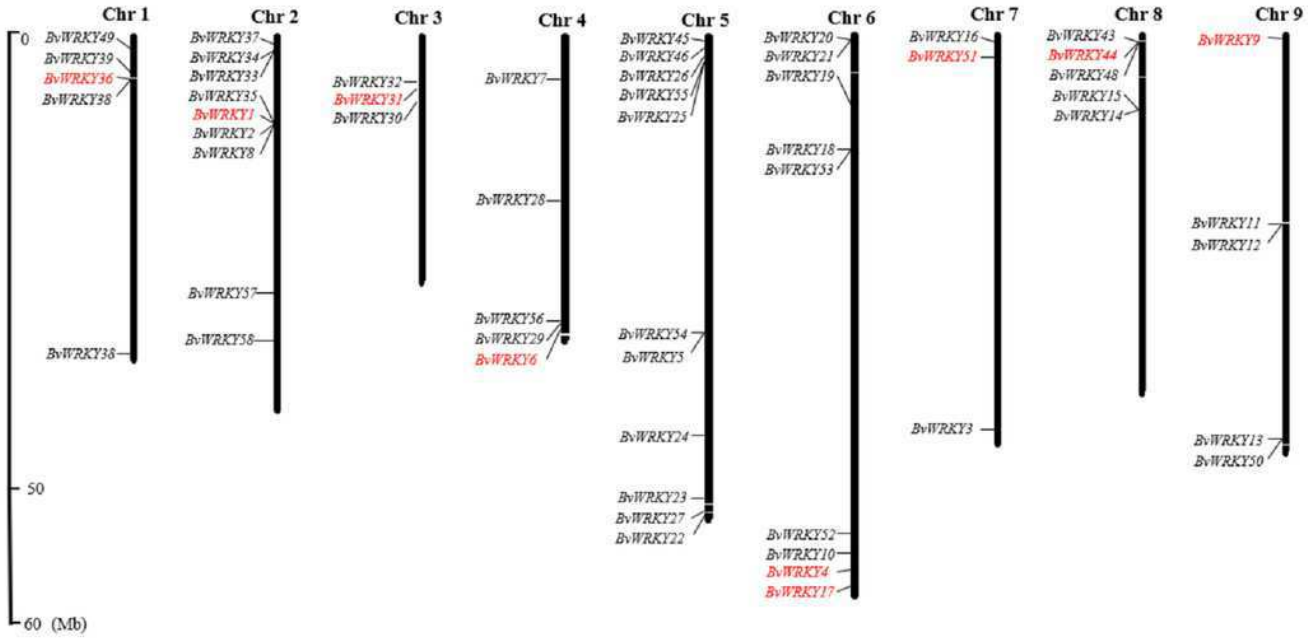

Figure 3

BvWRKY proteins motifs identified by MEME using the complete amino acid sequences of BvWRKY.

Combined *p*-values are indicated and different motifs were shown by different colors and numbered from 1 to 10. Detailed information of BvWRKY motifs was listed in Fig. S1.

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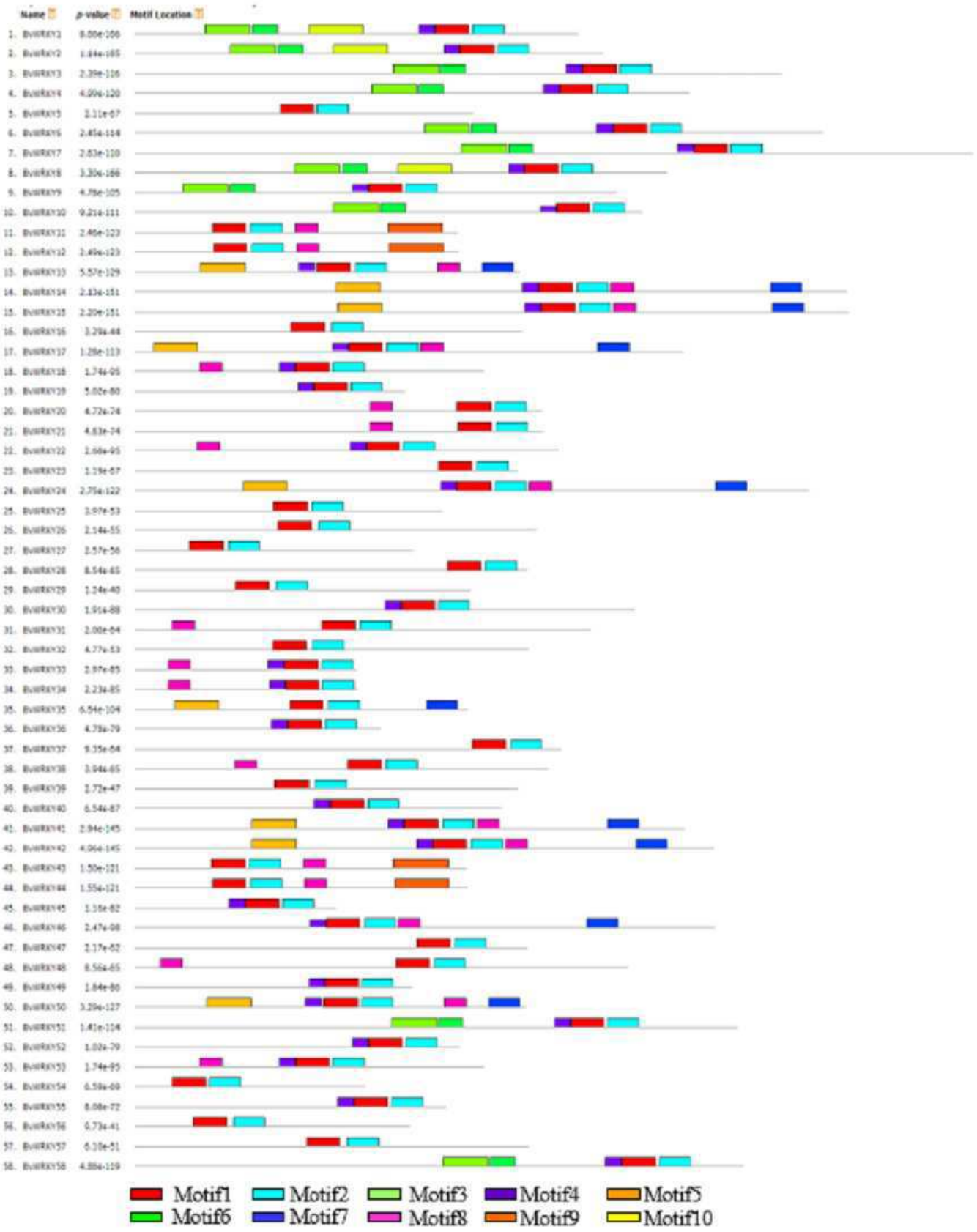

Figure 4

The exon-intron structure of *BvWRKY*genes according to the phylogenetic relationship.

The unrooted phylogenetic tree was constructed based on the full-length sequences of *BvWRKY* with 1,000 bootstraps. Exon-intron structure analyses of *BvWRKY* genes were performed by using the online tool GSDS. Lengths of exons and introns of each *BvWRKY* gene were exhibited proportionally. Introns are represented by black lines. Exons are represented by yellow boxes. Upstream/downstream are represented by blue boxes. The scale of genes length is given at the bottom. CDS: Coding sequence.

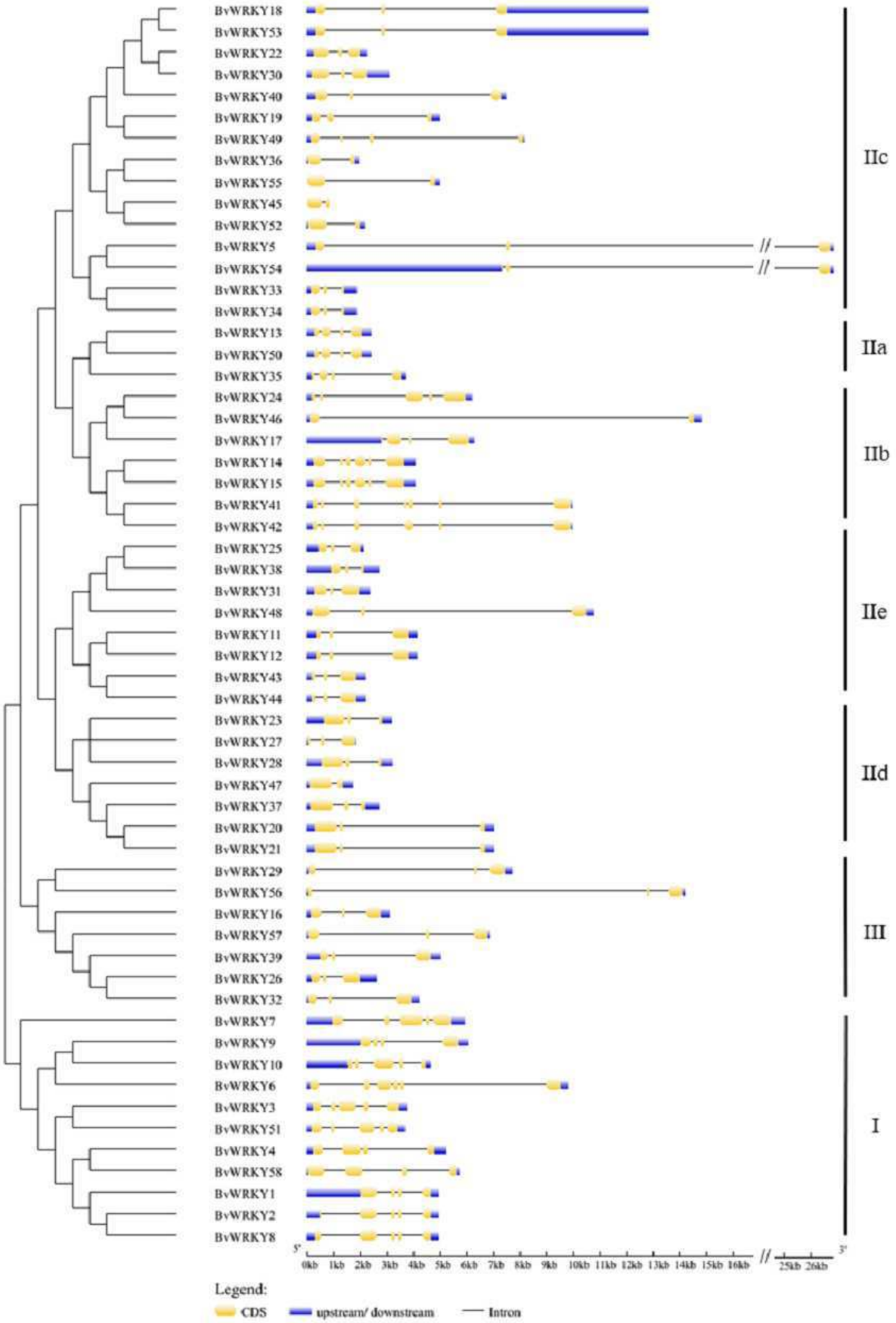

Figure 5

Relative expression levels of nine *BvWRKY*genes in shoot and root of sugar beet seedlings exposed to 0, 15, 25, 50, and100 mM $NaHCO_3$ for 72 h.

Expression of the *BvWRKY* genes normalized to those of *BvACTIN* and shown relative to the expression at 0 mM $NaHCO_3$. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression levels of target genes at different treatments. Experiments were repeated at least three times. Values are means ± SE and bars indicate SE (n = 3). Columns with different letters indicate significant differences at $P < 0.05$ (Duncan's test).

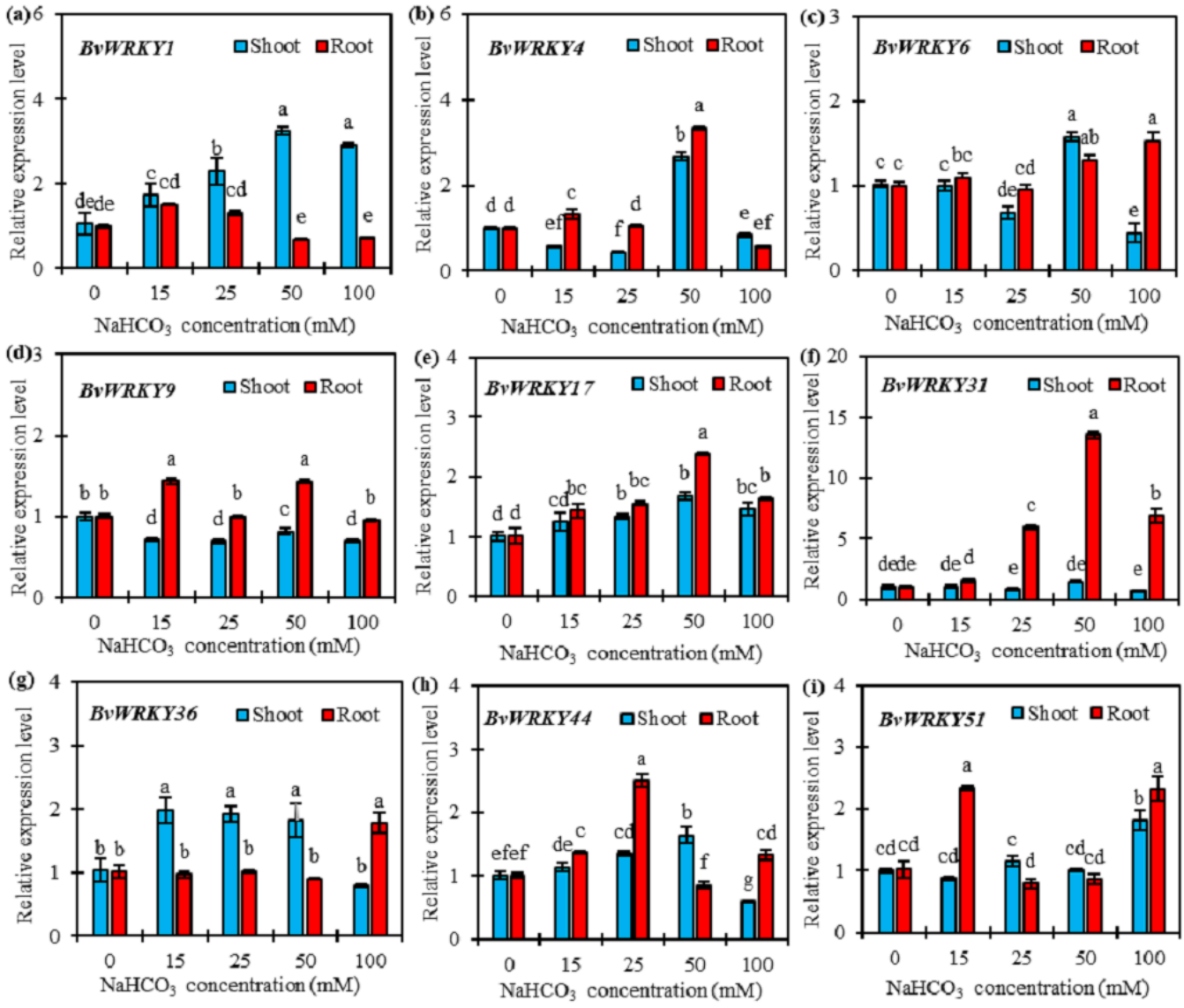

Table 1(on next page)

Sequences of primers used in qRT-PCR.

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1

No.	Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
1	*BvACTIN*	ACTGGTATTGTGCTTGACTC	ATGAGATAATCAGTGAGATC
2	*BvWRKY1*	CTCCAGATGATGTTCCAAGGACAC	GGCACAGCAAGAAAGAGAAGTG
3	*BvWRKY4*	CGGAAAATCTCACAACTCCCTCTTCT	TTCGGAGAAGAAACTCGAGACCAG
4	*BvWRKY6*	CTCAACCTAATCGCCGACTTC	ATTAAATGGAGGCACGCGGT
5	*BvWRKY9*	GCAGTGATTGTAGCTCCTAAGGTT	ATGGTTTCTCAGGGACAACAGA
6	*BvWRKY17*	GGAGACCGAGATCAGTGGTTCTTC	TACTTCTCCCATCTTTGCTTTGGC
7	*BvWRKY31*	CGGCTACCACTAGACTTAGCTCCT	GTCTTTAAGCTCATCTTGTGACGTGC
8	*BvWRKY36*	CCTCATGGATGAACTACAAAACGTCG	ATCAACGGCATCCGAAACGTTAATC
9	*BvWRKY44*	CTACCTCAAGCTAGCATGGAAGCAA	TCTTAGGAGATGATATGGAGGCGGC
10	*BvWRKY51*	GGCTCCTTCTTCACTTTCTGTCTC	CCACCAAATGCTCCTACAGTTG

2

Table 2(on next page)

Identification of *BvWRKY* genesusing sugar beet genome data.

1

Gene name	Accession No.	Chr	Exons count	CDS (bp)	ORF (aa)	MW (KD)	pI	Type
BvWRKY1	XM_010669274.2	2	6	1,221	406	55	9	I
BvWRKY2	XM_010669270.2	2	6	1,290	555	61.3	8.9	I
BvWRKY3	XM_010687642.2	7	5	1,782	593	65.6	7.2	I
BvWRKY4	XM_010684786.2	6	4	1,527	508	55.2	6.7	I
BvWRKY5	XM_010679791.2	5	4	933	310	34.8	7.7	IIc
BvWRKY6	XM_010677574.2	4	6	1,893	630	68.4	6	I
BvWRKY7	XM_010675633.2	4	6	2,307	768	84.2	5.9	I
BvWRKY8	XM_010669263.2	2	6	1,467	488	53.8	8.7	I
BvWRKY9	XM_010667792.2	9	5	1,326	441	48.4	5.8	I
BvWRKY10	XM_010684669.1	6	6	1,398	465	51.7	8	I
BvWRKY11	XM_010691421.2	9	3	891	296	32.1	5.4	IIe
BvWRKY12	XM_010691420.2	9	3	894	297	32.2	5.4	IIe
BvWRKY13	XM_010693285.2	9	4	1,062	353	39	6.9	IIa
BvWRKY14	XM_010688505.2	8	6	1,959	652	70.7	6.3	IIb
BvWRKY15	XM_010688504.2	8	6	1,965	654	70.9	6.3	IIb
BvWRKY16	XM_010685173.2	7	3	1,068	355	40.6	5.9	III
BvWRKY17	XM_010684943.2	6	5	1,509	502	55	8.5	IIb
BvWRKY18	XM_010682426.2	6	5	963	320	34.8	7	IIc
BvWRKY19	XM_010682026.2	6	3	744	247	28.5	9.2	IIc
BvWRKY20	XM_010681226.2	6	3	1,122	373	40.9	9.6	IId
BvWRKY21	XM_010681225.2	6	3	1,125	374	41	9.6	IId
BvWRKY22	XM_010681114.2	5	3	1,167	388	42.6	5.8	IIc
BvWRKY23	XM_010680880.2	5	3	1,056	351	38.7	9.7	IId
BvWRKY24	XM_010680272.2	5	5	1,857	618	68.1	6	IIb
BvWRKY25	XM_010678110.2	5	3	846	281	32	6.2	IIe
BvWRKY26	XM_010678017.2	5	3	1,107	368	41.4	5.8	III
BvWRKY27	XM_010681091.2	5	3	768	255	29.3	8.9	IId
BvWRKY28	XM_010676798.2	4	3	1,083	360	40.4	9.9	IId
BvWRKY29	XM_010677529.2	4	3	927	308	35.5	6.2	III
BvWRKY30	XM_010673459.2	3	3	1,377	458	50.3	6.3	IIc
BvWRKY31	XM_010673298.2	3	3	1,257	418	46.4	5.8	IIe
BvWRKY32	XM_010673610.2	3	3	1,086	361	40.7	6.2	III
BvWRKY33	XM_010671053.2	2	3	612	203	23.3	5.8	IIc
BvWRKY34	XM_010671052.2	2	3	615	204	23.4	5.4	IIc
BvWRKY35	XM_010669209.2	2	4	918	305	34.4	6.2	IIa
BvWRKY36	XM_010671487.2	1	2	678	225	25.8	6.4	IIc
BvWRKY37	XM_010690290.2	1	3	1,173	390	43.7	9.7	IId
BvWRKY38	XM_010671526.2	1	3	1,140	379	40.9	6.3	IIe
BvWRKY39	XM_010670954.2	1	3	1,056	351	39.2	6.4	III
BvWRKY40	XM_010668205.2	Un	3	1,011	336	37.3	6.3	IIc
BvWRKY41	XM_010667897.2	Un	6	1,515	504	55.5	5.6	IIb

BvWRKY42	XM_010667896.2	Un	6	1,593	530	58.6	5.5	IIb
BvWRKY43	XM_010687939.1	8	3	915	304	33.9	5.7	IIe
BvWRKY44	XM_010687938.1	8	3	918	305	34	5.7	IIe
BvWRKY45	XM_010677949.1	5	2	681	226	25.4	9.3	IIc
BvWRKY46	XM_010697085.2	5	2	558	185	21.3	9	IIb
BvWRKY47	XM_010694702.2	2	2	1,083	360	39.2	9.7	IId
BvWRKY48	XM_010667324.1	8	3	1,359	452	48.9	6.2	IIe
BvWRKY49	XM_010693736.1	1	4	765	254	29.2	7.8	IIc
BvWRKY50	XM_019251511.1	9	4	1,080	359	39.7	6.4	IIa
BvWRKY51	XM_019250725.1	7	5	1,659	552	61.5	5.8	I
BvWRKY52	XM_010684417.2	6	2	897	298	35.1	8.6	IIc
BvWRKY53	XM_019250206.1	6	5	963	320	34.8	7	IIc
BvWRKY54	XM_019249620.1	5	4	636	211	24	9.2	IIc
BvWRKY55	XM_010678106.2	5	2	858	285	32	9	IIc
BvWRKY56	XM_010677727.2	4	3	759	252	28.6	5.5	III
BvWRKY57	XM_010671801.2	2	3	1,086	361	39.9	6.7	III
BvWRKY58	XM_010671916.2	2	4	1,677	558	61.4	9.4	I

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