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GENOME-WIDE ANALYSIS OF bZIP FAMILY GENES IDENTIFIES THEIR STRUCTURAL DIVERSITY, EVOLUTIONARY PATTERNS AND EXPRESSION PROFILES IN RESPONSE TO SALT STRESS IN SUGAR BEET

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Abstract

Sugar beet is an important crop with significant biotechnological potential. The basic leucine zipper (bZIP) transcription factor family plays critical roles in salinity stress response in many plant species, and its investigation in sugar beet will assist in improving productivity under salt stress. Using bioinformatics software, the entire catalogue of sugar beet bZIP (BvbZIP) regular genes (45) and proteins (50) was critically examined, revealing a wide range of physicochemical properties suggesting functional versatility. Eleven phylogenetic groups were detected, based on relationships with *Arabidopsis thaliana*. *BvbZIP* genes with similar exon counts and BvbZIP proteins with similar motif compositions were typically clustered in the same phylogenetic class. Among the *BvbZIP* genes, ~94% (42) were assigned to their chromosomal locations and shown to have expanded primarily through segmental duplication. *In silico* gene expression analysis revealed a wide implication of *BvbZIPs* in the response to salinization, with 7 candidate *BvbZIPs* that were strongly up- or down-regulated in response to salt treatment in a salt-sensitive/tolerant cultivar, while maintaining a constant expression level in the other cultivar, implying that they play a role in sugar beet salt-responsive signalling network. We showed that these candidate genes exhibited considerable conservation with their sea beet counterparts, suggesting that they could be beneficial in enhancing sugar beet salinity tolerance. Our findings provide the first genome-wide view of the sugar beet *bZIP* gene family and lay the groundwork for deeper functional validation of selected candidate *bZIP* genes.

Key words: bZIP transcription factor family; Sugar beet; Comparative phylogeny; Expression profiling; *In silico* analysis; Salt stress; Candidate genes.

Introduction

Sugar beet (*Beta vulgaris* subsp. *vulgaris*) is a recently domesticated crop that is primarily cultivated in temperate climate zones. As of 2021, the global production of beet amounted to ~262 Million Tons from about 4.3 million ha harvested, with Russia, France, the USA, Germany and Turkey, as the world's leading sugar beet producers (https://knoema.com/atlas/topics/Agriculture). Sugar beet is an important source of sugar in the world, accounting for nearly 30% of the annual production of sugar (Dohm *et al*., 2014), as well as a source of animal feed and bioethanol (Zabed *et al*., 2014; Evans & Messerschmidt, 2017). It is a diploid species with 2n = 18 chromosomes, belongs to the family *Amaranthaceae*, and has a genome size estimated to 714–758 megabases. Sugar beet improvement has been developed to increase productivity, sugar content or other desirable traits for breeders (Monteiro *et al*., 2018). Nonetheless, environmental stress is responsible for sugar beet productivity and quality losses (Porcel *et al*., 2018). Soil salinization, in particular, is a major threat to agricultural output and has become a global environmental concern (Duarte *et al*., 2013). Salt stress affects more than 20% of the world's farmed land, and this number is growing by the day (Roy & Chowdhury, 2020). Although sugar beet is a salt-tolerant crop (Hossain *et al*., 2017; Skorupa *et al*., 2019), prolonged early growth stage salt stress has a negative influence on germination and seedling growth (Kaffka & Hembree, 2004). As a result, enhancing research on sugar beet salt tolerance at early development stages would markedly increase sugar yields in many irrigated areas.

Due to their sessile nature, plants cannot move to avoid unfavorable conditions, thus they have to cope with a variety of harsh environmental factors. To survive stressors, plants have evolved complex signaling transduction pathways and various stress tolerance mechanisms. Previous research has shown that transcription factors (TFs) play an essential role in plant stress response signaling by binding to the promoters of specific sets of stress-responsive genes to stimulate or suppress their expression (Chen *et al*., 2002; Jin *et al*., 2017). In the plant kingdom, at least 64 transcription factor families have been described (Perez-Rodriguez *et al*., 2010). Among these, seven major TF families have been linked to stress responses (Finkelstein & Lynch, 2000). The basic-region-leucine-zipper (bZIP) TF family is one of them, and it plays an important role in abiotic stress tolerance (Dröge-Laser *et al*., 2018). There is substantial evidence that *bZIP* genes are important regulators of abiotic stress-response signaling (Jakoby *et al*., 2002; Nijhawan *et al*., 2008; Fujita *et al*., 2012; Wei *et al*., 2012) and their functions in stress tolerance are typically realized via abscisic acid (ABA)-dependent pathway. It has been demonstrated that *bZIP* genes found in a variety of plant species play critical roles in salt stress response (Li *et al*., 2020; Wang *et al*., 2021; Kumar *et al*., 2021).

The bZIP TFs are named after their shared feature, the bZIP domain, which is ~ 60–80 amino acids long and includes two functional regions located on a contiguous alpha-helix: a strongly conserved basic region and a more diversified leucine zipper region (Vinson *et al*., 1989; Dröge-Laser *et al*., 2018). The basic region is positioned at the N-terminus of the bZIP domain and consists of an invariant N-x7-R/K motif with approximately 16 amino acid residues, which is responsible for DNA binding and nuclear localization (Ji *et al*., 2018). The leucine zipper region, positioned precisely nine amino acids towards the C-terminus, hosts a heptad repeat [$(g,a,b,c,d,e,f)_n$] of leucines and other hydrophobic amino acids and mediates the homo- and/or heterodimerization of bZIP proteins (Yang *et al*., 2019a). Besides the bZIP domain, bZIP

transcription factors have other conserved motifs to modulate their transcription-regulatory activity (Liu *et al*., 2014; Jakoby *et al*., 2002). For instance, two conserved motifs, R/KxxS/T and S/TxxD/E, have been confirmed as Ca^{2+} independent protein kinase and casein kinase II phosphorylation sites (Furihata *et al*., 2006).

So far, several members of the bZIP family have been identified and characterized using genome-wide analyses in a variety of monocot and dicot species, such as *Arabidopsis thaliana* (Jakoby *et al*., 2002; Deppmann *et al*., 2004; Dröge-Laser *et al*., 2018), rice (Nijhawan *et al*., 2008), maize (Wei *et al*., 2012), tomato (Li *et al*., 2015), apple (Zhao *et al*., 2016), strawberry (Wang *et al*., 2017), carrot (Que *et al*., 2015), wheat (Kumar *et al*., 2018; Agarwal *et al*., 2019) and radish (Fan *et al*., 2019). However, no reference genome-wide report is available for the *bZIP* gene family in sugar beet. The current study is the first genome-wide analysis of *bZIP* genes in sugar beet, including their conserved motifs, gene structure, chromosomal distribution and evolutionary relationships with their counterparts from *B. vulgaris* subsp. *maritima*, with an increased focus on their expression profiles under salt stress.

Material and Methods

Database retrieval and sequence filtering of bZIP family members in sugar beet and *Arabidopsis thaliana*: A total of 46 and 72 *bZIP* genes of *B. vulgaris* subsp. *vulgaris* and *A. thaliana,* respectively, were retrieved from the iTAK - Plant Transcription factor & Protein Kinase Identifier and Classifier (http://itak.feilab.net/cgi-bin/itak/index.cgi). Including isoforms, the *B. vulgaris* and *A. thaliana* genes encoded 51 and 120 proteins, respectively. The amino acid sequences obtained from both species were further examined with the Pfam database version 32.0 (https://pfam.xfam.org/) to confirm the presence and integrity of the bZIP domain (PF00170, PF07716 or PF03131). Protein sequences with an irregular or incomplete bZIP domain were excluded from the study. Thus, only 50 and 119 protein sequences were considered as candidates of BvbZIPs and AtbZIPs, respectively. Proteins from *B. vulgaris* subsp. *vulgaris* and *A. thaliana* were given the systematic names BvbZIPs and AtbZIPs, respectively (Table 1). Among sugar beet bZIP regular proteins, BvbZIP3-1 and BvbZIP3-2 were isoforms derived from the same gene (*BvbZIP3*). The same was true for BvbZIP18-1/18-2/18-3, BvbZIP27-1/27-2, and BvbZIP40-1/40-2. From the two examined species, 49 BvbZIPs and 118 AtbZIPs had a typical bZIP domain, with an invariant N-× 7-R/K motif in the basic region and a heptad repeat positioned exactly nine amino acids toward the C terminus. For gene structure analysis, gene sequences of all *BvbZIPs* were extracted from the sugar beet genome (NCBI Bioproject accession PRJNA41497).

bZIP protein physico-chemical features, alignment and phylogenetic analysis: The molecular weight (MW), isoelectric point (pI), grand average of hydropathicity (GRAVY), number of amino acids, and percentage of positively/negatively-charged residues of the BvbZIP amino acid sequences were determined using the Expert Protein Analysis System (ExPASy) program (http://web.expasy.org/protparam/) and EMBOSS Pepstats (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/). Furthermore, we used the WoLF PSORT tool https://wolfpsort.hgc.jp/) to predict the subcellular location of BvbZIP proteins. The amino acid sequences of the bZIP genes were imported into the MEGA5.2 (Tamura *et al*., 2011) software and multiple sequence alignments were performed with ClustalW. Following that, an unrooted neighbour-joining (NJ) phylogenetic tree was generated using 1,000 bootstrap replications and the Jones-Taylor-Thornton (JTT) model based on the alignment data.

Gene structure, motif composition and genome distribution of the BvbZIPs: The exon-intron organizations of the *BvbZIP* genes were visualized using Gene Structure Display Server (GSDS 2.0; http://gsds.cbi.pku.edu.cn/) by aligning the cDNAs with their corresponding genomic DNA sequences. Additionally, the MEME online program version 5.1.1 (http://meme-suite.org/tools/meme) was employed to detect supplementary motifs beyond the bZIP domain. The number of motifs was limited to 10, and the motif widths were set between 20 and 80. The remaining parameter settings were kept to default.

To determine the chromosomal locations of bZIP genes in the sugar beet genome, locus coordinate information was obtained from the *Beta vulgaris* Resource (http://bvseq.boku.ac.at/). The physical chromosome map was, then, generated using MapInspect software (Available online at: http://mapinspect.software.informer.com/), and the *BvbZIP* genes were graphically displayed on the *B. vulgaris* chromosomes. The *bZIP* gene pairs resulting from segmental or tandem duplication were linked by lines. The *bZIP* genes with tandem duplication events were defined as adjacent homologous genes on a single chromosome, while segmental duplications were defined as duplication events occurring between different chromosomes (Liu *et al*., 2011).

Expression profiling of sugar beet *bZIP* genes and comparative analysis of *bZIP* genes between sugar beet and sea beet: To better understand the expression patterns of the sugar beet *bZIP* gene family under salt stress, we used RNA-sequencing (RNA-Seq) data generated by Geng *et al*., (2019) for two sugar beet cultivars, namely a salt-sensitive cultivar, S710, and a salt-tolerant cultivar, T710MU. Experimentally, Geng *et al*., (2019) subjected a group of seedlings from both cultivars, with uniform growth, to salt stress (1/2 × Hoagland solution with 280 mmol/L NaCl) for 15 days, along with control seedlings (0 mmol/L NaCl). Following this, they systematically identified and characterized the salt-sensitive mRNAs expressed in the seedlings of each cultivar (S710 and T710MU) at a transcriptome-wide scale, using Illumina HiSeq PE150 high-throughput sequencing. From the whole transcriptome expression data of each cultivar, we retrieved the 'fragments per kilo base of transcript per million mapped fragments' (FPKM) values of the set of *BvbZIP* genes studied here (control and treatment of each cultivar), as well as the log2 fold change values which were used to generate the *BvbZIP* genes expression heat map using the Clustergrammer web-based tool (https://maayanlab.cloud/clustergrammer/) with "Euclidean distance" for the distance measurement and "Average linkage" as the clustering method.

Table 1. Regular bZIP family protein sequences of *Beta vulgaris* and *Arabidopsis thaliana* retrieved from iTAK - Plant Transcription factor & Protein Kinase Identifier and Classifier (http://itak.feilab.net/cgi-bin/itak/index.cgi) and used in this study.

iTAK ID	Systematic ID	iTAK ID	Systematic ID
	***Beta vulgaris* bZIP proteins (BvbZIPs)**		
Bv_005360_xxys.t1	BvbZIP1	Bv3_063410_ixst.t1	BvbZIP23
Bv_011410_ndfg.t1	BvbZIP2	Bv3_064480_rnwf.t1	BvbZIP24
Bv_012640_iicn.t1	BvbZIP3-1	Bv3_070510_azad.t1	BvbZIP25
Bv_012640_iicn.t2	BvbZIP3-2	Bv4_081830_goqc.t1	BvbZIP26
Bv1_003640_tkfj.t1	BvbZIP4	Bv4_086160_cqyx.t1	BvbZIP27-1
Bv1_005070_wokp.t1	BvbZIP5	Bv4_086160_cqyx.t2	BvbZIP27-2
Bv1_006910_rqak.t1	BvbZIP6	Bv4_094850_mmdf.t1	BvbZIP28
Bv1_011350_yiue.t1	BvbZIP7	Bv5_107170_psah.t1	BvbZIP29
Bv1_011670_nwdc.t1	BvbZIP8	Bv5_121770_gprg.t1	BvbZIP30
Bv1_013750_smoy.t1	BvbZIP9	Bv6_129360_qxwi.t1	BvbZIP31
Bv1_021380_gmre.t1	BvbZIP10	Bv6_134230_aref.t1	BvbZIP32
Bv2_025990_gnmo.t1	BvbZIP11	Bv6_135660_ocpz.t1	BvbZIP33
Bv2_028580_zsnk.t1	BvbZIP12	Bv6_140280_ckch.t1	BvbZIP34
Bv2_035120_znqm.t1	BvbZIP13	Bv6_140290_joxm.t1	BvbZIP35
Bv2_041830_atex.t1	BvbZIP14	Bv7_159570_afnu.t1	BvbZIP36
Bv2_042510_dwuo.t1	BvbZIP15	Bv7_159880_nyaj.t1	BvbZIP37
Bv2_042860_ixzz.t1	BvbZIP16	Bv7_169340_mcdu.t1	BvbZIP38
Bv3_050990_kqjn.t1	BvbZIP17	Bv7_175790_dsuf.t1	BvbZIP39
Bv3_052640_ymhm.t1	BvbZIP18-1	Bv7_176150_dhtu.t1	BvbZIP40-1
Bv3_052640_ymhm.t2	BvbZIP18-2	Bv7_176150_dhtu.t2	BvbZIP40-2
Bv3_052640_ymhm.t3	BvbZIP18-3	Bv8_186270_jcpj.t1	BvbZIP41
Bv3_053880_mpkc.t1	BvbZIP19	Bv9_207080_uszh.t1	BvbZIP42
Bv3_057700_ifwp.t1	BvbZIP20	Bv9_209240_nxdc.t1	BvbZIP43
Bv3_060650_iwdw.t1	BvbZIP21	Bv9_214060_hwek.t1	BvbZIP44
Bv3_062960_chhy.t1	BvbZIP22	Bv9_224390_wsiw.t1	BvbZIP45
	***Arabidopsis thaliana* bZIP proteins (AtbZIPs)**		
AT1G03970.1	AtbZIP1	AT3G10800.1	AtbZIP35
AT1G06070.1	AtbZIP2	AT3G12250.1	AtbZIP36-1
AT1G06850.1	AtbZIP3-1	AT3G12250.2	AtbZIP36-2
AT1G06850.2	AtbZIP3-2	AT3G12250.3	AtbZIP36-3
AT1G08320.1	AtbZIP4-1	AT3G12250.4	AtbZIP36-4
AT1G08320.2	AtbZIP4-2	AT3G12250.5	AtbZIP36-5
AT1G08320.3	AtbZIP4-3	AT3G17609.1	AtbZIP37-1
AT1G13600.1	AtbZIP5	AT3G17609.2	AtbZIP37-2
AT1G19490.1	AtbZIP6	AT3G17609.3	AtbZIP37-3
AT1G22070.1	AtbZIP7	AT3G17609.4	AtbZIP37-4
AT1G32150.1	AtbZIP8	AT3G19290.1	AtbZIP38-1
AT1G42990.1	AtbZIP9	AT3G19290.3	AtbZIP38-3
AT1G43700.1	AtbZIP10	AT3G30530.1	AtbZIP39
AT1G45249.1	AtbZIP11	AT3G44460.1	AtbZIP40
AT1G49720.1	AtbZIP12-1	AT3G49760.1	AtbZIP41
AT1G49720.2	AtbZIP12-2	AT3G51960.1	AtbZIP42-1
AT1G59530.1	AtbZIP13	AT3G51960.2	AtbZIP42-2
AT1G68640.1	AtbZIP14	AT3G54620.1	AtbZIP43-1
AT1G68880.1	AtbZIP15	AT3G54620.2	AtbZIP43-2
AT1G75390.1	AtbZIP16-1	AT3G54620.3	AtbZIP43-3

Table 1. (Cont'd.).

iTAK ID	Systematic ID	iTAK ID	Systematic ID
AT1G75390.2	AtbZIP16-2	AT3G56660.1	AtbZIP44
AT1G77920.1	AtbZIP17	AT3G56850.1	AtbZIP45
AT2G04038.1	AtbZIP18	AT3G58120.1	AtbZIP46
AT2G12900.1	AtbZIP19	AT3G62420.1	AtbZIP47
AT2G12940.1	AtbZIP20	AT4G01120.1	AtbZIP48
AT2G13150.1	AtbZIP21	AT4G02640.1	AtbZIP49-1
AT2G16770.1	AtbZIP22	AT4G02640.2	AtbZIP49-2
AT2G17770.2	AtbZIP23	AT4G34000.1	AtbZIP50-1
AT2G18160.1	AtbZIP24	AT4G34000.2	AtbZIP50-2
AT2G21230.1	AtbZIP25-1	AT4G34000.3	AtbZIP50-3
AT2G21230.2	AtbZIP25-2	AT4G34590.1	AtbZIP51
AT2G21230.3	AtbZIP25-3	AT4G35040.1	AtbZIP52
AT2G22850.1	AtbZIP26-1	AT4G35900.1	AtbZIP53
AT2G22850.2	AtbZIP26-2	AT4G36730.1	AtbZIP54-1
AT2G31370.1	AtbZIP27-1	AT4G36730.2	AtbZIP54-2
AT2G31370.2	AtbZIP27-2	AT4G37730.1	AtbZIP55
AT2G31370.3	AtbZIP27-3	AT4G38900.1	AtbZIP56-1
AT2G31370.4	AtbZIP27-4	AT4G38900.2	AtbZIP56-2
AT2G31370.5	AtbZIP27-5	AT4G38900.3	AtbZIP56-3
AT2G31370.6	AtbZIP27-6	AT5G06839.1	AtbZIP57-1
AT2G35530.1	AtbZIP28	AT5G06839.2	AtbZIP57-2
AT2G36270.1	AtbZIP29	AT5G06839.3	AtbZIP57-3
AT2G40620.1	AtbZIP30	AT5G06950.1	AtbZIP58-1
AT2G40950.1	AtbZIP31	AT5G06950.2	AtbZIP58-2
AT2G41070.1	AtbZIP32-1	AT5G06950.3	AtbZIP58-3
AT2G41070.2	AtbZIP32-2	AT5G06950.4	AtbZIP58-4
AT2G41070.3	AtbZIP32-3	AT5G06960.1	AtbZIP59-1
AT2G42380.1	AtbZIP33-1	AT5G06960.2	AtbZIP59-2
AT2G42380.2	AtbZIP33-2	AT5G07160.1	AtbZIP60
AT2G46270.1	AtbZIP34-1	AT5G08141.1	AtbZIP61
AT2G46270.2	AtbZIP34-2	AT5G10030.1	AtbZIP62-1
AT3G10800.1	AtbZIP35	AT5G10030.2	AtbZIP62-2
AT3G12250.1	AtbZIP36-1	AT5G11260.1	AtbZIP63
AT3G12250.2	AtbZIP36-2	AT5G15830.1	AtbZIP64
AT3G12250.3	AtbZIP36-3	AT5G24800.1	AtbZIP65
AT3G12250.4	AtbZIP36-4	AT5G28770.1	AtbZIP66-1
AT3G12250.5	AtbZIP36-5	AT5G28770.2	AtbZIP66-2
AT3G17609.1	AtbZIP37-1	AT5G28770.3	AtbZIP66-3
AT3G17609.2	AtbZIP37-2	AT5G38800.1	AtbZIP67
AT3G17609.3	AtbZIP37-3	AT5G42910.1	AtbZIP68
AT3G17609.4	AtbZIP37-4	AT5G44080.1	AtbZIP69
AT3G19290.1	AtbZIP38-1	AT5G60830.1	AtbZIP70
AT3G19290.3	AtbZIP38-3	AT5G65210.1	AtbZIP71-1
AT2G41070.3	AtbZIP32-3	AT5G65210.2	AtbZIP71-2
AT2G42380.1	AtbZIP33-1	AT5G65210.3	AtbZIP71-3
AT2G42380.2	AtbZIP33-2	AT5G65210.4	AtbZIP71-4
AT2G46270.1	AtbZIP34-1	AT5G65210.5	AtbZIP71-5
AT2G46270.2	AtbZIP34-2	AT5G65210.6	AtbZIP71-6

In order to evaluate the conservation of the selected *bZIP* genes between sugar beet and sea beet, we conducted a BLASTn search of the BvbZIP CDS sequences against the *B. vulgaris* subsp. *maritima* genome, available in the *Beta vulgaris* resource (http://bvseq.boku.ac.at/). The nucleotide and protein sequences of the BvbZIP and BmbZIP orthologous genes were subjected to sequence alignment using Clustal W (https://www.genome.jp/tools-bin/clustalw). The degree of conservation of each orthologous gene pair was calculated as the number of matches over the number of pairwise alignments (Shabalina *et al*., 2004).

Results and Discussion

Physicochemical properties of BvbZIP TFs: The *BvbZIP* genes differed significantly in terms of the size and sequences of their encoded proteins, as well as their physicochemical properties. For the 50 predicted BvbZIP proteins, the amino acid (aa) content ranged from 141 to 690, with an average of 343 aa. The proteins' deduced molecular weights varied from 16.21 to 75.23 KDa. These BvbZIPs had a wide range of isoelectric focusing points; eleven (11) BvbZIP proteins out of 50 identified were shown to have pI >7 and the remaining 39 proteins had pI <7. BvbZIP16 had the highest pI (9.9), while BvbZIP9 had the lowest pI of 4.78. All 50 BvbZIP proteins had negative GRAVY values, indicating that they were hydrophilic. Furthermore, the percentage of aliphatic amino acids (23.63%) was approximately three-fold higher than that of aromatic amino acids (7.48%) in BvbZIP proteins, suggesting that the BvbZIP proteins were rich in aliphatic amino acids. On the other hand, there was no discernible disparity in the proportion of positively and negatively charged amino acids in BvbZIPs (11.24% and 12.33%, respectively). The overall wide range in the physicochemical properties of BvbZIPs was similar to *bZIP* genes from other plant species (Liu & Chu, 2015), reflecting their probable functional diversity. For the subcellular location, all BvbZIP proteins could be predicted to a nuclear localization, comparable to sweet potato, pomegranate and wheat (Yang *et al*., 2019a; Liang *et al*., 2022; Wang *et al.,* 2022). In other species, such as *Fagopyum talaricum*, *Cucumis sativus* L. and *Gossypium hirsutum*, some *bZIP* genes had subcellular locations other than the nucleus, such as the endoplasmic reticulum, chloroplast and mitochondrion, indicating that the *bZIP* genes are likely to regulate biological processes in organelles (Liu *et al*., 2019; Baloglu *et al*., 2014; Wang *et al*., 2020).

Phylogenetic relationships of BvbZIP proteins: An unrooted Neighbor-Joining tree was built using the amino acid sequences of 50 proteins from sugar beet and 119 proteins from *Arabidopsis*. As shown in (Fig. 1), the phylogenetic tree clustered all bZIP members into 13 groups, which were named A, B, C, D, E, F, G, H, I, J, K, M and S, according to the classification of *A. thaliana* bZIP family proposed by Dröge-Laser *et al*., (2018). This classification system has been endorsed for other species depending on the clustering of *bZIP* genes from their own and *Arabidopsis* genomes (Wei *et al*., 2012; Liu *et al*., 2014; Pourabed *et al*., 2014; Liu &Chu 2015; Li *et al*., 2015; Hu *et al*., 2016a; Zhao *et al*., 2016).

Among all groups, S and D were the largest, each including 9 members of BvbZIP proteins. Group A that followed groups S and D in the number of sequences, contained 7 members in sugar beet. Groups G and I (Ia+Ib) had six (6) members each. The remaining groups lacked a significant number of BvbZIPs. Groups C, E and F, for example, each had 3 members. Group H contained 2 members. Finally, both groups K and B were the smallest groups in *B. vulgaris*, each containing only one BvbZIP. Interestingly, groups J and M included only AtbZIP6 and AtbZIP60, with no BvbZIP members. Likewise, there were no subfamily M bZIPs found in *Chenopodium quinoa* (Li *et al*., 2020), and no subfamily J bZIPs were found in *Amaranthus hypochondriacus* (Li *et al*., 2020) or potato (Wang *et al*., 2021).

When the number of groups in sugar beet was compared to other plant species, it was noticed that the sugar beet bZIP family had the same number of groups as *Salvia miltiorrhiza* (Zhang *et al*., 2018), Tartary buckwheat (Liu *et al*., 2019), banana (Hu *et al*., 2016b), apple (Zhao *et al*., 2016) and maize (Wei *et al*., 2012), fewer groups than watermelon and cotton, which both had 13 groups (Yang *et al*., 2019b; Wang *et al*., 2020), but more groups than sesame, grapevine, tomato and cucumber, which had 9, 10, 9 and 6 groups, respectively (Baloglu *et al*., 2014; Liu *et al*., 2014; Li *et al*., 2015; Wang *et al*., 2018).

The phylogeny of *BvbZIP* gene family may serve for predicting their stress-related roles. For example, it was reported that some group D members in *A. thaliana* act as crucial transcriptional regulators in systemic acquired resistance (SAR) (Dröge-Laser *et al*., 2018). As for group A, there are many lines of evidence supporting the important role of this group in abiotic stress response and ABA signaling pathways (Dröge-Laser *et al*., 2018). Members of group G in *A. thaliana* were widely involved in the control of light-responsive promoters; however, their possible involvement in cellular defense against pathogens and abiotic stress was also proposed (Jakoby *et al*., 2002; Dröge-Laser *et al*., 2018). Other studies linked group I bZIPs to stress response, cell cycle regulation and various developmental aspects (Dröge-Laser *et al*., 2018).

The present study identified several orthologs to *BvbZIP* genes from the model plant *A. thaliana* (Fig. 1), which may be helpful to infer putative functions of *BvbZIP* genes. As is well known, orthologous genes are likely to retain the same function (Tatusov *et al*., 1997). For example, the ortholog of BvbZIP7 gene in *A. thaliana* is the *AtbZIP10* (*VIP1*) gene (AT1G43700.1), which regulates stress-related genes by binding to VIP1 response elements (VRE) (Lacroix & Citovsky, 2013). Also, the *A. thaliana* ortholog of *BvbZIP9* gene is *AtbZIP9* (AT1G42990.1), which acts during endoplasmic reticulum stress (ER) by activating unfolded protein response (UPR) target genes *via* direct binding to the UPR element (UPRE) (Dröge-Laser *et al*., 2018). The orthologous gene of *BvbZIP36* is *AtbZIP11* (AT1G45249.1), which is involved in abscisic acid (ABA) and stress responses and acts as a positive component of glucose signal transduction (Kim *et al*., 2004).

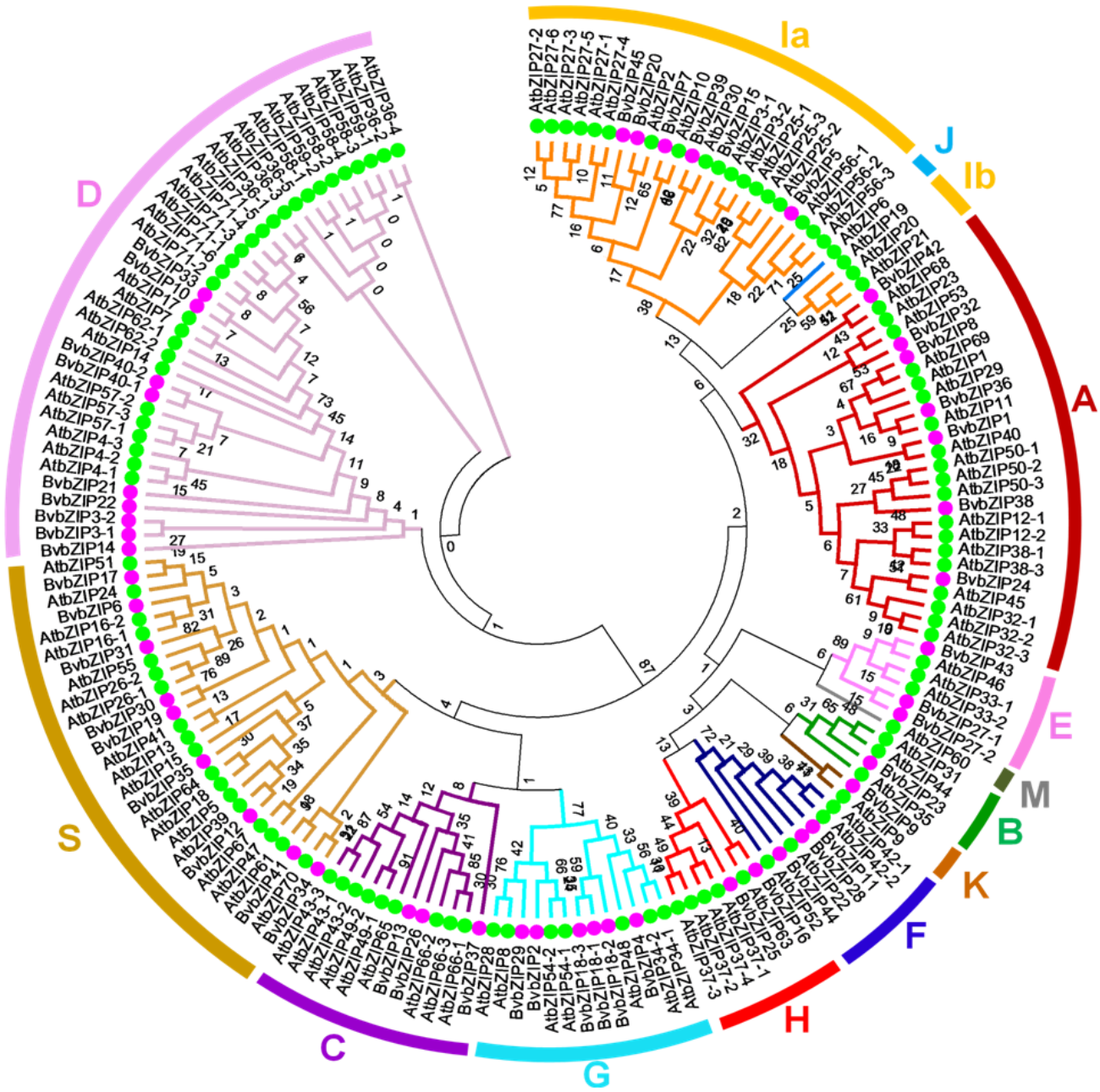

Fig. 1. Unrooted phylogenetic tree depicting the relationships between bZIP proteins of sugar beet (*Beta vulgaris*) and *Arabidopsis thaliana*. Sugar beet (*BvbZIPs*: pink filled circles) and *Arabidopsis* (*AtbZIP*: green filled circles) genes are indicated at the end of branches. The various colored arcs represent different groups of bZIP proteins (Groups A–K, M and S), which were named based on *A. thaliana* phylogeny (Dröge-Laser *et al*., 2018).

Gene structure and motif composition of BvbZIP proteins: Each gene's structure may be a signature that documents the gene's emergence and evolution within a family (Betts *et al*., 2001). So, it was intriguing to discover the gene structure of *BvbZIPs* in order to gain a better understanding of their evolutionary history. Gene structure analyses in several plant species revealed that the majority of genes in the same group had similar exon-intron structure and exon numbers (Liu *et al*., 2014; Hu *et al*., 2016b; Liu *et al*., 2019; Yang *et al*., 2019b). Our findings showed that the *BvbZIP* genes contained exons ranging from 1 to 12, and that most members within the same group shared a similar intron/exon organization, indicating their close evolutionary relationship.

As shown in Fig. 2, all *BvbZIP* genes belonging to the groups S and F were intronless. Similar cases have also been reported in *Arabidopsis* (Dröge-Laser *et al*., 2018), maize (Wei *et al*., 2012), banana (Hu *et al*., 2016b), watermelon (Yang *et al*., 2019b) and apple (Zhao *et al*., 2016). The remaining *BvbZIPs* contained exons in numbers varying from 2 to 12. The average number of exons in group G was 11.5, followed by group D with an average number of exons of 10.5. All genes of group C had 6 exons, and those from groups E, I and H had 4 exons. The average number of exons in group A was 3.4 and several members (4/7) of this group contained 3 exons, except for *BvbZIP8*, *BvbZIP36* and *BvbZIP38*, each with four exons. Group K contained 3 exons and finally group B contained 2 exons. These results revealed that gene structure

was highly conserved across the phylogenetic clades, lending support to the group classification. In addition to the intronless genes of groups S and F, groups B and K, as well as half of members of group A (4/7 genes) had no more than two introns, which provided support to the hypothesis that a low number of introns was probably related to stress response (Zhao *et al*., 2016; Zhou *et al*., 2018). On the other hand, groups G and D had significantly more introns than the other groups, which was consistent with the results from previous studies (Hu *et al*., 2016a; Hu *et al*., 2016b). A previous report on rice found that the rate of intron loss was faster than the rate of intron gain following segmental duplication (Nuruzzaman *et al*., 2010). It is possible that groups G and D possess the original exons, through which the exons in the other groups were generated via gene duplication with subsequent intron loss. Moreover, exon/intron gain/loss was also observed between paralogous genes within the same group. For example, *BvbZIP29* had 12 exons, whereas its paralogous *BvbZIP2* contained 11 exons, suggesting that one exon was lost during sugar beet genome evolution. These gains and losses could be caused by chromosomal rearrangements and fusions, and they have the potential to create functional diversity in a number of gene families (Xu *et al*., 2012).

Using MEME program, a total of 10 conserved motifs were identified from all the BvbZIP proteins (Fig. 3). Members with equivalent motif compositions were clustered into the same group, confirming their evolutionary closeness. This feature of the bZIP conserved motifs has also been observed in grapevine (Liu *et al*., 2014), cassava (Hu *et al*., 2016a) and banana (Hu *et al*., 2016b). As shown in Fig. 3, motif 1, which is found in all the BvbZIP TFs, has been annotated as bZIP domain. Aside from the bZIP domain, some additional conserved motifs were shared by several groups, including motif 4 in groups S, G, C, E, I and D; motif 7 in groups G, E and B; motif 8 in groups A, E and D; motif 9 in groups S, E, I, and F; and motif 10 in groups A, E, I and D. Other conserved motifs, on the other hand, appeared in particular groups, and thus they may assign specialized roles to members of these groups. For example, motifs 2 and 3 were found only in group D, while motifs 5 and 6 were found only in group S. Strikingly, groups H and K were devoid of any specific motifs, suggesting that the BvbZIPs of these two groups might have limited functionality in comparison to the remaining BvbZIP family members.

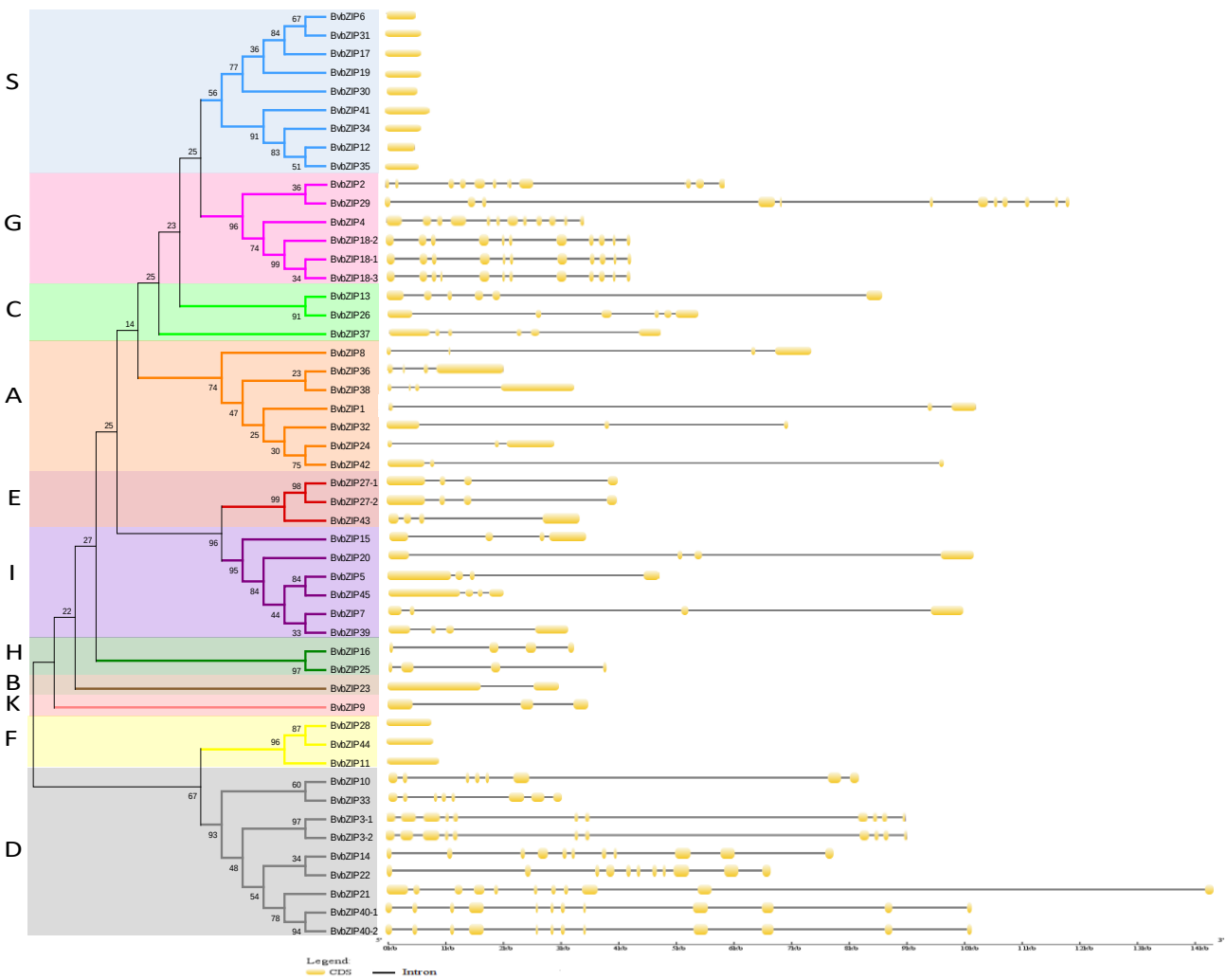

Fig. 2. Exon-intron structure of *BvbZIP* genes according to evolutionary relationships. The phylogenetic tree was built, using MEGA 5.2 software, from a complete alignment of 50 BvbZIP proteins by the Neighbour-Joining method with bootstrapping analysis (1 000 replicates). Sugar beet *bZIP* genes are indicated at the end of branches. Lines connecting two exons represent introns.

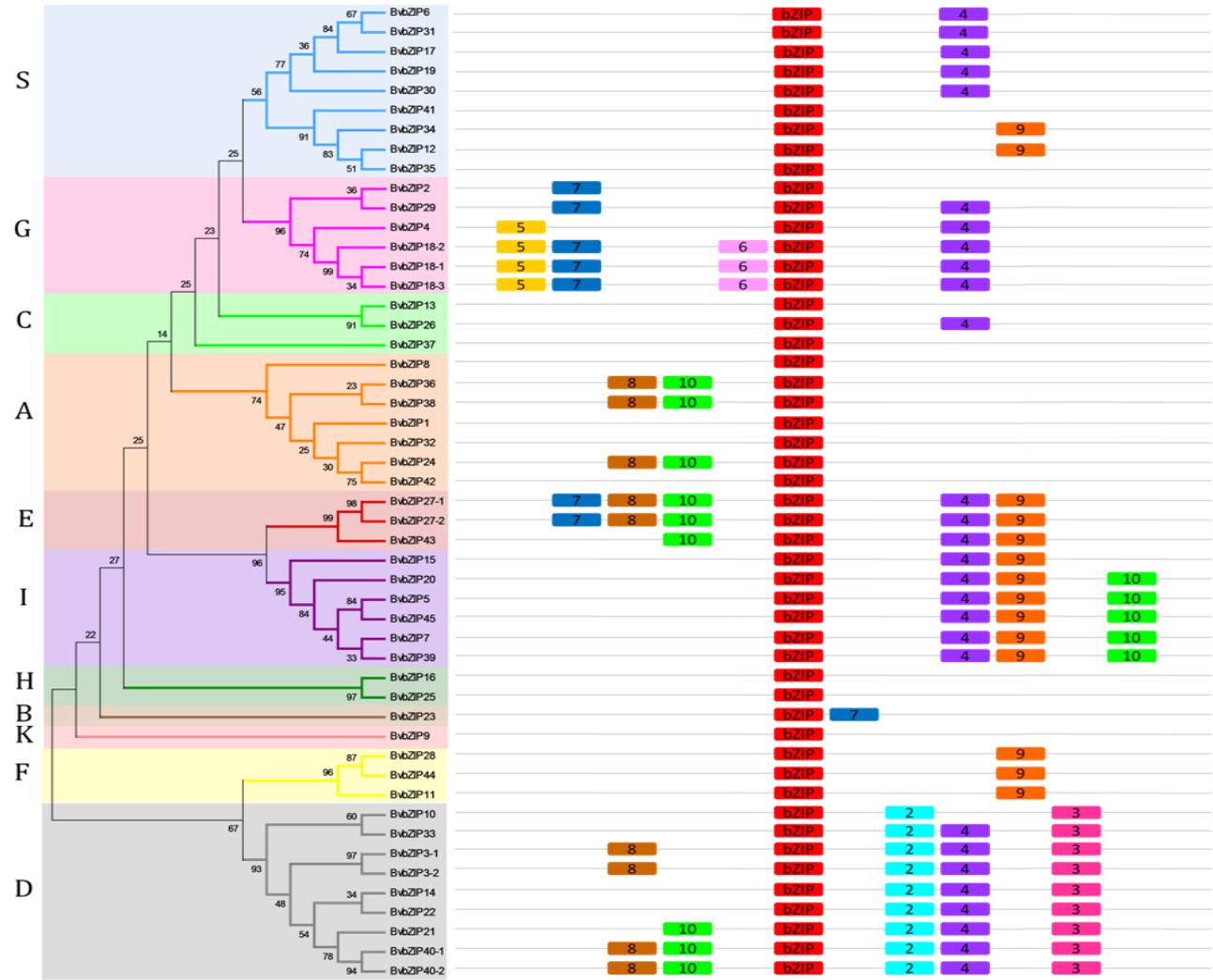

Fig. 3. Phylogenetic relationships and architecture of conserved protein motifs in sugar beet *bZIP* genes. The phylogenetic tree was constructed using MEGA 5.2 software, based on the full-length sequences of sugar beet bZIP proteins. Sugar beet *bZIP* genes are indicated at the end of branches. Clusters are depicted in various colors. Ten conserved motifs (bZIP and 2–10), are shown in various colored boxes.

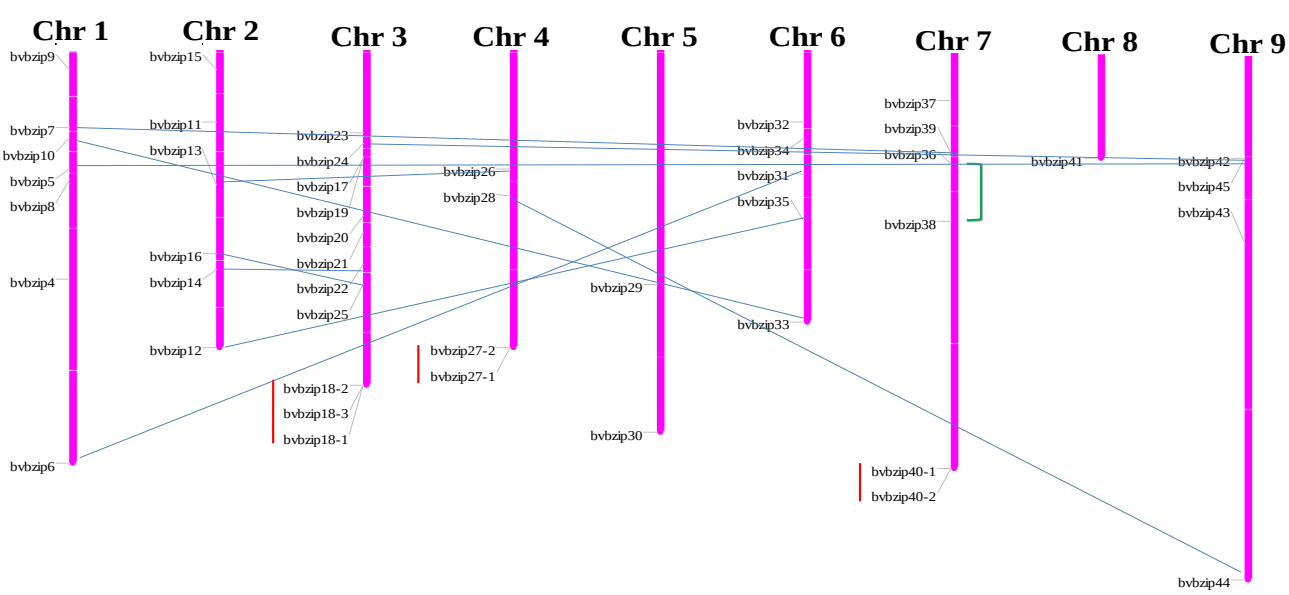

Fig. 4. Chromosomal location and duplication events of sugar beet *bZIP* genes (*BvbZIP*s). Each chromosome is labelled with its chromosome number at the top. The gene names correspond to the chromosomal locations of the sugar beet *bZIP* genes. The blue and green lines connect the paralogous gene pairs in duplicated blocks. Only one pair of paralogous genes (*BvbZIP36*/*BvbZIP38*) was classified as tandem duplication (green line), while the others were segmental duplication (blue lines). The vertical red lines show different transcripts derived from a single gene (isoforms).

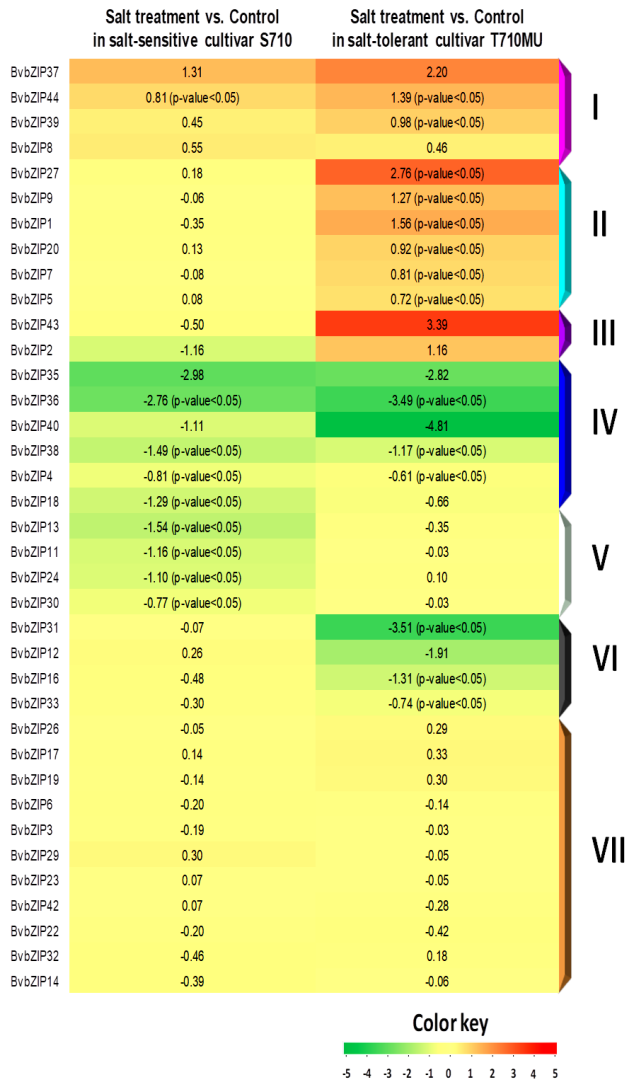

Fig. 5. Heatmap depicting expression changes of *BvbZIP* genes when a salt-sensitive (S710) and salt-tolerant (T710MU) cultivars were submitted to salt stress (280 mM) at early seedling stage, based on transcriptome sequencing (Geng *et al*., 2019). The log2 fold change values of *BvbZIP* genes were used to generate a heat map. Green denotes under-regulation and red denotes up-regulation in treatment (280 mM), in comparison with control (0 mM). *BvbZIP10*, *BvbZIP25*, *BvbZIP28* and *BvbZIP45* are not shown in the heatmap because of missing expression data (FPKM). Unexpressed/very weakly expressed genes (*BvbZIP15*, *-21*, *-34* and *-41*) are also not shown.

Except for motifs 2 and 5, which were annotated by Pfam as MFMR (multifunctional mosaic region) and DOG1 (delay of germination) domains, the biological functions of the conserved motifs found in BvbZIPs proteins were mostly unknown. The DOG1 domain was found in all of the group D members, and it had been reported that this domain was required for dormancy induction and various phases of seed maturation, in part by interfering with ABA signaling modules (Nishimura *et al*., 2018). Moreover, the MFMR motif was observed in 4 members of group G. The N-terminal half of MFMR motif is rather rich in proline residues and has been termed the Proline-rich domain "PRD" (Siberil *et al*., 2001), and some of these motifs may play a role in protein-protein interactions (Meier & Gruissem, 1994). Interestingly, these two motifs (DOG1 and MEMR) have also been identified in bZIPs from the same groups (D and G) in other plant genomes, such as watermelon (Yang *et al*., 2019b), strawberry (Wang *et al*., 2017) and barley (Pourabed *et al*., 2014), suggesting that the functions of these groups have been conserved across plant species during evolution. Overall, these findings showed that bZIP members from the same phylogenetic cluster were characterized by close motif compositions, indicating the presence of shared functional patterns within each subfamily.

Genome distribution and gene duplication events of sugar beet bZIP family: To investigate the chromosomal distribution of *BvbZIP* genes, the physical map of the *BvbZIP* members was drawn, based on the genomic position data obtained from the *Beta vulgaris* resource (http://bvseq.boku.ac.at/). Except for *BvbZIP1*, *-2*, and *-3*, the identified 45 *BvbZIP* genes, were distributed on all chromosomes of sugar beet (Fig. 4). *BvbZIP1* and *BvbZIP2* were anchored on unmapped scaffolds 0139.scaffold00419 and 0316.scaffold00796, respectively, whereas *BvbZIP3* (a single gene encoding two protein isoforms, *BvbZIP3-1* and *BvbZIP3-2*), was anchored on 0390.scaffold00899. Interestingly, the distributions of the remaining 42 *BvbZIP* genes were not even. Chromosome 3 contained the highest number of *BvbZIP* genes (9 genes), followed by chromosomes 1, which had seven genes. There were six *bZIP* genes on chromosome 2; five *bZIP* genes on each of chromosomes 6 and 7, four genes on chromosome 9 and three genes on chromosome 4. Finally, chromosomes 5 and 8 had two genes and one gene, respectively.

We explored genome duplication events, including tandem and segmental duplications, to better understand the evolutionary mechanisms of *BvbZIP* gene family expansion. Gene duplications are important events driving gene family expansion and have played a critical role in protein functional diversification throughout plant evolution (Zhou *et al*., 2018; Fan *et al*., 2019; Yang *et al*., 2019b). Only one pair of tandem duplication (*BvbZIP36*/*BvbZIP38*) was detected on chromosome 7 (Fig. 4). This could imply that tandem duplication played only a minor role in the expansion of the *BvbZIP* gene family. In contrast, we found 10 pairs of paralogous genes randomly scattered across the genome, which were considered to be evidence of segmental duplication. Overall, our findings suggested that segmental duplication events were primarily responsible for the expansion of the *bZIP* gene family in sugar beet, and therefore, were the principal driving force for the evolution of this gene family. A similar pattern of duplication mechanisms was discovered in the bZIP family in rice (Nijhawan *et al*., 2008), grapevine (Liu *et al*., 2014) and sesame (Wang *et al*., 2018).

Table 2. Nucleotide and protein sequences identities of *BvbZIP* and *BmbZIP* orthologous genes.

***Beta vulgaris* bZIPs and their *B. maritima* orthologs**	**CDS length**	**Identities (%)**	**Protein length**	**Identities (%)**
BvbZIP9	918	915/924 (99.02%)	305	304/307 (99.02%)
mar_g6190.t1	924		307	
BvbZIP11	810	803/ 810(99.13%)	269	265/269 (98.51%)
mar_g19115.t1	810		269	
BvbZIP13	1041	1028/1041(98.75%)	346	341/346 (98.55%)
mar_g19269.t1	1041		346	
BvbZIP16	507	505/507 (99.60%)	168	168/168 (100%)
mar_g18272.t1	507		168	
BvbZIP24	1002	997/1017(98.03%)	333	333/338 (98.52%)
mar_g3854.t1	1017		338	
BvbZIP27-1	1059	1049/1059(99.05%)	352	350/ 352 (99.43%)
mar_g12662.t2	1056		351	
BvbZIP27-2	1062	1052/1062(99.05%)	353	351/ 353 (99.43%)
mar_g12662.t1	1059		352	
BvbZIP31	543	532/543 (97.97%)	180	177/180 (98.33%)
mar_g25060.t1	537		178	

***BvbZIP* gene expression patterns under salt stress in salt-sensitive and salt-tolerant cultivars:** Evidence suggests that *bZIP* genes are implicated in saline stress response and related signal transduction pathways in a variety of plants (Yu *et al*., 2020; Wang *et al*., 2018; Li *et al*., 2020; Wang *et al*., 2021; Kumar *et al*., 2021). Nonetheless, there was no genome-wide documentation of the response of *bZIP* genes to this stimulus in sugar beet. Using the transcriptome data from the study of Geng *et al*., (2019), we analyzed the transcription profiles of the *BvbZIP* genes in two sugar beet cultivars; a salt-sensitive cultivar (S710) and a salt-tolerant one (T710MU).

Qualitatively, the two sugar beet cultivars responded to salt stress via a differential gene regulation scheme. Different expression patterns were observed in seven expression clusters (Fig. 5). Cluster I genes were found to be up-regulated in the two sugar beet cultivars. Members of cluster II were not differentially expressed in the salt-sensitive cultivar but were significantly up-regulated in the salt-tolerant one. Cluster III contained two members that were down-regultaed in the salt-sensitive cultivar, but up-regulated in the salt-tolerant cultivar. Six members of cluster IV were significantly downregulated in the two sugar beet cultivars. Cluster V contained four members that were significantly down-regulated in the salt-sensitive cultivar (p-value<0.05), while remaining constant in the salt-tolerant cultivar. Cluster VI contained four members that were differentially expressed under salt treatment (down-regulated) in the salt-tolerant cultivar only. Finally, Cluster VII contained 11 members with no discernible differential expression in either cultivar. Although salinity stimulated or inhibited the expression of *BvbZIPs* from clusters I and IV, respectively, they would not be involved in the salinity tolerance signalling network because there was no variation in expression between the salt-sensitive and salt-tolerant cultivars.

Seven interesting gene expression patterns (*BvbZIP9*, *BvbZIP11*, *BvbZIP13*, *BvbZIP16*, *BvbZIP24*, *BvbZIP27*, and *BvbZIP31*) were identified using quantitative gene expression log2 fold change and p-value. *BvbZIP9* and *BvbZIP27*, both belonging to cluster II, maintained a constant quantitative expression after salt treatment in the salt-sensitive cultivar, whereas their expression was significantly increased (p-value < 0.05, fold change >1) in salt-tolerant T710MU. In cluster V, *BvbZIP11*, *BvZIP13* and *BvbZIP24* showed a significant decrease in expression (p<0.05; fold change > 1) after the salt-sensitive S710 was treated with 280mM NaCl, whereas salt treatment had no effect on their expression levels in the salt-tolerant T710MU. Finally in cluster VI, *BvbZIP31* and *BvbZIP16* exhibited constant expression levels in the salt-sensitive cultivar in both control (0 mM NaCl) and treatment (280 mM) but were substantially under-expressed in salt-tolerant T710MU (from ~134 FPKM to only ~11 FPKM, and from ~16 FPKM to ~6 FPKM for *BvbZIP31* and *BvbZIP16*, respectively; p-value < 0.05). Importantly, the expression profiles of the identified candidate genes, *BvbZIP9*, *BvbZIP11*, *BvbZIP13*, *BvbZIP16*, *BvbZIP24*, *BvbZIP27* and *BvbZIP31* should be more deeply analyzed by qRT-PCR in commercial sugar beet varieties, which would help elucidate salt-tolerance gene regulatory networks in relevant cultivars.

The candidate genes approach based on RNA-Seq data is a powerful tool for quickly accessing a collection of expressed sequences that can be used to develop functional markers within differentially-expressed genes themselves (Salgotra *et al*., 2020). Such functional markers would improve the selection efficiency aimed at developing varieties with desired traits. In recent years, a number of functional markers have been developed and used in marker-assisted breeding programs, successfully enhancing quality traits in various crops such as sorghum (Too *et al*., 2018), wheat (Zhang *et al*., 2014), maize (Lubberstedt *et al*., 2005) and rice (Lau *et al*., 2015).

Comparative analysis of *bZIP* genes induced by salt stress between sugar beet and its highly salt-tolerant relative, sea beet: Although sugar beet is a highly salt-

tolerant crop that withstands high salt stress better than other plants species (Rozema *et al*., 2015; Hossain *et al*., 2017; Skorupa *et al*., 2019), its tolerance to high salinity is reduced compared to sea beet, *B. vulgaris* subsp. *maritima*. A genome assembly of sea beet is hosted in the *Beta vulgaris* resource and it could provide a good comparative resource for *BvbZIP* genes involved in salt stress responsiveness. As a result, the coding (CDS) and protein sequences (including isoforms) of seven *B. maritima BmbZIP* genes, orthologous to *BvbZIP* genes *BvbZIP9*, *-11*, *-13*, *-16*, *-24*, *-27*, and *-31)* were retrieved from the sea beet genome and proteome. The alignment results, shown in Table 2, revealed a high conservation of the nucleotide and protein sequences. The nucleotide sequence's identity ranged from 97.97% to 99.60% with an average of 98.82%. The protein sequence conservation varied between 98.33% and 100% identity, with an average value of 98.97%. With such high sequence conservation, orthologous proteins in both beet species may perform the same activities in salt tolerance. This conservation is expected because it is believed that all sugar beet modern cultivars descended from sea beet (Biancardi *et al*., 2012), as modern sugar beet selection likely began in Germany, Austria, and Italy, in the late 1800s/early 1900s, from fodder beet × *B. maritima* hybrids, which were performed to increase the sugar content trait in fodder beet.

Conclusions

Our study revealed that the sugar beet genome potentially encoded 50 regular bZIP proteins that were structurally diverse and could be classified into 11 phylogenetic groups. Such a phylogenetic classification of *BvbZIPs* was supported by exon-intron gene structures as well as conserved motifs, and we report that *BvbZIPs* have been evolutionarily expanded primarily via segmental duplications. Although sugar beet is a salt-tolerant crop, prolonged early growth stage salt stress has a negative influence on germination and seedling growth, and one major group targeted for stress tolerance is the bZIP transcription factor family. Expression profiling of *BvbZIP* full-genome genes, based on RNA-Seq public transcriptome data led to the identification of seven candidate *BvbZIP* genes that were highly up- or down-regulated in response to salt treatment in a salt-sensitive/salt-tolerant cultivar, while retaining a steady expression level in the other cultivar. We showed that these candidate genes exhibited considerable conservation with their sea beet counterparts, suggesting that they could be beneficial in enhancing sugar beet salinity tolerance. Overall, the findings of this study provided a wealth of information, identified candidate genes, and opened the door to future experimental validation, as well as the use of *BvbZIP* candidates in genetic improvement programs.

Acknowledgments

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