Identification and characterization of the BZR transcription factor family and its expression in response to abiotic stresses in *Zea mays* L.

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Abstract Brassinosteroids (BRs) are plant specific steroidal hormones that play diverse roles in regulating a broad spectrum of plant growth and developmental processes, as well as, in responding to various biotic and abiotic stresses. Extensive research over the years has established stress-impact-mitigating role of BRs and associated compounds in different plants exposed to various abiotic and biotic stresses, suggesting the idea that they may act as immunomodulators, thus opening new approaches for plant resistance against hazardous environmental conditions. In this research the characterization of the transcriptional response of 11 transcription factors (TFs) belonging to BRASSINAZOLE-RESISTANT 1 (BZR1) TF family of *Zea mays* L. was analyzed in seedlings subjected to different stress conditions. Being important regulators of the BR synthesis, BZR TFs might have stress resistance related activities. However, no stress resistance related functional study of BZR TFs has been reported in maize so far. *In silico* analyses of the selected 11 TFs validated the features of their protein domains, where a highest degree of similarity observed with recognized BZR TFs of rice and *Sorghum bicolor*. Additionally, we investigated the organ-specific expression of 11 *ZmBZR* in maize seedlings. Five of them did not show any transcript accumulation, suggesting that *ZmBZR* expression might be regulated in a manner dependent on plant developmental stage. For the remaining six *ZmBZR*, their ubiquitous expression in the whole plant indicates they could function as growth regulators during maize development. More importantly, in response to various stress conditions, the spatial transcript accumulation of all *ZmBZR* varies along the plant. All six *ZmBZR* showed up-regulation against N starvation, hypoxia and salt stress. On the contrary, heat stress clearly down-regulated gene expression of all *ZmBZR* analysed. Consistently with the expression results, the distribution of stress-related *cis*-acting elements in the promoter of these genes inferred that the maize BZR TFs might play some roles in regulating the expression of the corresponding genes in response to multifarious stresses. In conclusion, these data reveal that BZR TFs have stress signaling activity in maize, in addition to their confirmed role in regulating plant physiology and morphology.

Keywords: abiotic stress · brassinosteroids · BZR · gene expression · TFs · *Zea mays* L.

Abbreviations: BRs · brassinosteroids; TFs, transcription factors; BES, BRASSINOSTEROID-INSENSITIVE 1-EMS-SUPPRESSOR; BZR, BRASSINAZOLE-RESISTANT; Zm, *Zea mays* L.; RT-qPCR, reverse transcription quantitative real-time PCR

Introduction

Brassinosteroids (BRs) are plant specific steroidal hormones that play diverse roles in regulating a broad spectrum of plant growth and developmental processes. They regulate multiple physiological functions including seed germination, cell elongation and division, senescence, vascular-differentiation, reproduction, root development, photomorphogenesis and respond to various biotic and abiotic stresses (Saini et al. 2015; Singh and Savaldi-Goldstein 2015). Molecular studies evidenced cross-talk between BRs and other phytohormones and hypothesised the existence of synergistic effects between exogenous BR treatments and endogenous levels of other hormones (Gruszka 2013; Zhu et al. 2013). Extensive research over the years has led to the idea that BRs could act as stress-impact-mitigating compounds in different plants exposed to various abiotic stresses such as high temperature, low temperature in terms of chilling and freezing, salinity, light, drought, metals/metalloids and organic pollutants (Vardhini and Anjum 2015 and references therein). Some studies also suggest that BR treatments could promote plant resistance against many pathogens, such as fungi, bacteria, and virus (He et al. 2007; Kemmerling et al. 2007; Chinchilla et al. 2009). Essentially, BRs seem to act as immunomodulators when applied at the appropriate concentration and at the correct stage of plant development, thus opening new approaches for the improvement of plant resistance against hazardous environmental conditions.

Most of the information about BR signalling has been obtained from *Arabidopsis*. Molecular studies have demonstrated that BRs are perceived at the cell membrane by the BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor kinase, which upon ligand binding heterodimerizes with BRI1-ASSOCIATED RECEPTOR KINASE (BAK1). The fully activated BRI1/BAK1 triggers a series of downstream phosphorylation events and subsequently inactivates the GSK3/Shaggy-like protein kinase BIN2, a pivotal negative regulator of BR signaling (Li et al. 2001), which lead to the regulation of the expression of a large set of genes involved in plant growth and development (Sun et al. 2010). Downstream, BZR1 (BRASSINAZOLE RESISTANT1) and BES1 (BRASSINOSTEROID INSENSITIVE 1-ETHYL METHANESULFONATE-SUPPRESSOR 1), two closely related TFs belonging to the BRASSINAZOLE-RESISTANT (BZR) TF family, are rapidly dephosphorylated by protein phosphatase 2A (PP2A) (Tang et al. 2011). The dephosphorylated BZR1 and BES1 accumulate in the nucleus and directly bind to *cis* elements, known as E-box (CANNTG) and BR-response element (CGTGT/CG) of their target, regulating plant growth and development (Yu et al. 2011). Although the interaction between stress and BRs has long been observed (Nawaz et al. 2017), the underlying molecular mechanisms were far to be completely elucidated.

The BZR TF family appears to be involved in the regulation of various processes in plants. In *Arabidopsis*, BZR family proteins were thought to be the primary transcription factors regulating huge numbers of genes involved in BR signal output (Sun et al. 2010; Yu et al. 2011). Rice BZR family has been suggested to play a conserved role as in *Arabidopsis* (Tong and Chu 2012). Recent findings reveal that AtBZR1 positively regulates plant stress tolerance (Sahni et al. 2016); in *Brassica rapa*, BrBZR TFs family is suggested to be involved in regulating stress-related activities (Saha et al. 2015). While major studies have revealed the positive roles of these TFs in BR signal transduction in many plants (Yin et al. 2005), no genome-wide-in-depth study of the BZR TF family in maize has previously been reported.

In this work a comprehensive genome-wide analysis was carried out to characterize the BZR TFs family in maize. Eleven BZR TFs of *Zea mays* L. (ZmBZR) were characterized from a genome-wide survey and their expression profiles were assessed in different tissues. Considering that crop plants are subjected to combinations of abiotic stresses during their lifespan that greatly reduce productivity and that recent research suggests plants can be primed by chemical compounds to better tolerate different abiotic stresses, we proposed to better elucidate the role of BRs in stress response to test their effects in plant chemical priming. Being important regulators of the BR synthesis, BZR TFs might have

stress resistance related activities. However, no stress resistance related functional study of BZR TFs has been reported in the monocot model plant *Zea mays* L. so far. The expression analyses on the candidate *ZmBZR* were evaluated to investigate their responses to several abiotic stresses such as low nitrate availability, hypoxia, salinity and heat. The obtained results provide a new start for the future studies of the BR signalling pathway in monocotyledons.

Materials and Methods

Genome-wide identification of *ZmBZR* genes

To identify BZR TFs family members in *Zea mays* L., the *Arabidopsis* BZR1 amino acid sequence was used as query to search the maize Database (Phytozome). The conserved domains of the BZR were confirmed by Pfam (http://pfam.xfam.org). The list of genes analysed is reported in Supplemental Table S1, together with the primers utilized for reverse-transcription quantitative real-time PCR (RT-qPCR) expression analysis. Primers were designed with Primer3 web tool (version 0.4.0; http://frodo.wi.mit.edu/primer3/) and further verified with the PRATO web tool (http://prato.daapv.unipd.it). GRASSIUS database (http://grassius.org/) was used for gene nomenclature.

Phylogenetic analysis and classification of *ZmBZR* genes

The full amino acid sequences of BZR TFs members from maize, rice, sorghum, *Nicotiana* and *Arabidopsis* were aligned by CLUSTALW program. The gene IDs of BZR members in maize, rice, sorghum, *Nicotiana* and *Arabidopsis* are shown in Supplemental Table S2. Maize *BZR* genes were placed on 10 maize chromosomes according to their positions given in the GRAMENE maize database (available online: http://www.gramene.org). The distribution of *ZmBZR* genes on the maize chromosomes was drawn by MapInspect (available online: http://mapinspect.software.informer.com) and modified manually with annotation.

Cis-elements in the promoter regions of *ZmBZR* genes

To predict *cis*-acting regulatory DNA elements (*cis*-elements) in promoter regions of maize *BZR* genes, 2000 bp genomic DNA sequences upstream of the initiation codon (ATG) was analyzed by the PLACE website (available online: http://www.dna.affrc.go.jp/PLACE/signalscan.html).

Plant materials and growth conditions

Seeds of maize (*Zea mays* L.), inbred line B73, were washed in distilled water and germinated on wet filter paper at 25°C in the dark. After 3 days, maize seedlings were transferred in a controlled environmental chamber in 500 ml tanks containing a Hoagland-modified nutrient solution (changed every 2 days), according to the following composition (μM): KNO_3 (1000), $CaCl_2$ (200), $MgSO_4$ (200), KH_2PO_4 (40), FeNaEDTA (10), H_3BO_3 (4.6), $MnCl_2$ (0.9), $ZnCl_2$ (0.09), $CuCl_2$ (0.036), $NaMoO_4$ (0.01). This nutrient solution and a day/night cycle of 14 h/10 h at 25°C/20°C air temperature, 70/90% relative humidity, and 280 μmol m^{-2} s^{-1} photon flux density were utilized as standard conditions to grow control plants for each treatment. Four different stress treatments were imposed on maize seedlings: (i) nutritional, (ii) hypoxic, (iii) salt and (iv) heat stress. For nutritional stress, seedlings were grown in a nitrogen-depleted

nutrient solution (KNO_3 derived from the nutrient solution supplied to the control plants was replaced by 1 mM KCl). Hypoxic stress conditions were achieved by not bubbling air through the liquid solution for the entire experiment. For salt stress, a 100 mM NaCl concentration, which corresponds to severe salt stress in maize (Farooq et al. 2015; Henry et al. 2015; Zörb et al. 2015), was employed. Finally, an intense heat stress, generally greater than 4°C above optimum that in the case of maize is 25°C (Hatfield and Prueger 2015), was performed by growing seedlings in a day/night cycle at 35°C/30°C air temperature. After 5 days, control and treated plants were harvested by cutting the seedlings in four different parts (as illustrated in Fig. 5A), immediately frozen in liquid nitrogen and kept at -80°C for subsequent RNA extraction. An average of 20 randomly selected seedlings were used per sample in each experiment. Each experiment was repeated in triplicate.

RNA extraction and cDNA synthesis

Total RNA was extracted from 250 mg frozen tissue using the TRIzol method (Invitrogen, San Giuliano Milanese, Italy). Subsequently, an aliquot of total RNA was treated with RQ1 RNAse-free DNAse (Promega, Milano, Italy). Total RNA (1 µl) was quantified using a Nanodrop 1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA). Finally, cDNA was synthesized from 500 ng of total RNA mixed with 1 µl of 10 µM oligo-dT, as described by Trevisan et al. (2011).

Reverse-transcription quantitative real-time PCR (RT-qPCR)

Relative quantification of transcripts by RT-qPCR was performed in a StepOne Real-Time PCR System (Applied Biosystems, Monza, Italy). Reactions were performed using SYBR Green chemistry (Applied Biosystems), following the manufacturer's instructions. Reverse-transcribed RNA (2.5 ng) was used as template in each reaction as indicated by Manoli et al. (2014). Melting-curve analysis confirmed the absence of multiple products and primer dimers. Data were exported and analysed according to the method of Livak and Schmittgen (2001) and MIQE guidelines (Bustin et al. 2009), using *MEP* (membrane proteinPB1A10.07c, primers: forward 5'-TGTACTCGGCAATGCTCTTG-3' and reverse 5'-TTTGATGCTCCAGGCTTACC-3'), as the reference gene (Manoli et al. 2012). Only transcripts showing amplification with quantification cycle (C_q) < 35 were selected for subsequent gene expression analysis.

Results

Sequence analysis and phylogenetic classification

In order to identify *BZR* genes in *Zea mays* L. genome (B 73 RefGen_V3), the *Arabidopsis* BZR protein sequences were used as query to perform a genome-wide search. As a result, a total of 11 *BZR* genes were identified in the phytozome database (Table 1).

142 The predicted sizes of the 11 ZmBZR TFs ranged from 139 to 651 amino acids, and the predicted isoelectric points
143 varied from 5.11 to 10.7. The major domains of the 11 ZmBZR proteins were identified by Pfam (Punta et al. 2012).
144 Results showed that all BZR proteins possessed BZR signature that is essential for their activity as transcription factors.
145 Two proteins exhibited also a glycoside hydrolase catalytic domain.

146 **Chromosomal location, gene structure, and motif analysis of CPPs in maize**
147

148 A physical map was drawn to show the distribution of *ZmBZR* on different chromosomes of maize (Fig. 1). The 11
149 putative *ZmBZR* gene candidates were distributed across 7 of the 10 chromosomes in the maize genome. Among them,
150 chromosome 3 had three *ZmBZR* genes. Two *ZmBZR* genes were located on each of chromosomes 4 and 7. One *ZmBZR*
151 gene was situated on chromosomes 1, 2 and 9. Chromosomes 6, 8 and 10 do not included any *ZmBZR* genes.
152 Additionally, the DNA sequences of the 11 ZmBZR TFs were determined based on the *Z. mays* L. whole-genome
153 sequence. Analysis of the intron and exon distribution showed that most of the genes exhibited similar splicing patterns
154 (Fig. 2). A BLAST search of the NCBI database to compare the 11 ZmBZRs with BZRs of other species revealed that
155 the deduced amino acid sequences of ZmBZRs shared the highest similarity levels with other monocots, as rice and
156 *Sorghum bicolor* BZR TFs. The sequence similarity ranged from 50 to 94%, more specifically, 3 ZmBZRs shared
157 greater than 80% similarity with rice BZR TFs. The similarity among the ZmBZR TFs sequences ranged from 57 to
158 96%, and 11 BrBZRs shared greater than 80% similarity within the species, indicating their probable duplication (Fig.
159 3).
160 The aminoacidic sequences of *Arabidopsis*, rice, sorghum BZR TFs from NCBI were retrieved to construct a
161 phylogenetic tree with 11 deduced amino acid sequences of ZmBZR using the NJ method (Fig. 3). In this analysis, the
162 close homologs of *Arabidopsis* BZR and BZR-homolog (BEH) were included, but they resulted distant from all the
163 maize accessions studied. Four ZmBZR TFs formed a tight group with rice BZR1. The remaining five BZR TFs were
164 closely grouped in a separate part of the phylogenetic tree and exhibited distant relationships with both *Arabidopsis*
165 BZR TFs and the other ZmBZR. This result suggests that expansion of BZR1 and BZR2/BES1 took place after the
166 divergence of dicots and monocots. The presence of BZR TFs specific for monocots could also be assumed.
167 The subcellular *in silico* localization of the 11 ZmBZR TFs were carried out by Protcomp 9.0 from Softberry. As
168 transcription factor family proteins, 10 ZmBZRs were identified to have nuclear localization (Table 1). ZmBZR4 was
169 predicted to be located in plastid of maize cells. Three ZmBZR (ZmBZR1, 5 and 7) were predicted to be located in both
170 nucleus and plastid. Interestingly, the elements having a role in transcript localization were the CAT-box (cis-acting
171 regulatory element related to meristem expression), Motif I (cis-acting regulatory element root specific) and Skn-
172 1_motif (cis-acting regulatory element required for endosperm expression).

173 ***Cis*-acting elements analysis**
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175 Phytohormones such as auxin, ethylene, abscisic acid (ABA), gibberellins (GAs) and jasmonic acid (JA), are involved
176 in various processes throughout plants to overcome stress conditions. To identify the putative cis-acting regulatory
177 elements in ZmBZR TFs, about 2000-bp of the gene CDS, from the protein start codons (ATG) were analyzed by
178 PLACE database. The results showed that the *ZmBZR* genes contain various resistance- and hormone-related cis-acting
179 elements (Fig. 4). Many key cis-elements that were related to environmental stress signal responsiveness were
180 identified, such as MBS (MYB binding site, involved in drought-inducibility), TC-rich repeats (defence and stress-
181 responsive element), HSE (heat shock element), LTR (low temperature-responsive element) and several light

responsive elements such as G-box, Sp1, GAG-motif, and ACE (Fig. 4). Furthermore, cis-elements involved in phytohormone signaling, such as ABRE (abscisic acid-responsive element), ERE (ethylene-responsive element), TCA-element (salicylic acid-responsive element), CGTCA-motif (MeJA-responsive element), TGACG-motif (MeJA-responsive element), and P-box (gibberellin-responsive element) were also identified. *ZmBZR* genes also contained elements contributing to tissue-specific expression, including meristem specific elements (CCGTCC-box, CAT-box, CCGTCC-box, OCT) seed elements (RY-element), endosperm specific elements (GCN4_motif, Skn-1-like motif), trichome differentiation elements (MBSI) and vascular expression elements (AC-I, AC-II). Moreover, *ZmBZR* genes contained other functional elements, such as light-responsive elements and circadian control elements.

ZmBZR gene expression analyses

Expression analysis under unstressed conditions

Five of the 11 selected *ZmBZR1* genes (*ZmBRZ2*, *ZmBRZ3*, *ZmBRZ6*, *ZmBRZ7* and *ZmBRZ8*) were discarded since they evidenced very low amounts of transcripts in maize seedlings (data not shown). Therefore, the subsequent expression analyses were carried out on the remaining *ZmBZR* genes (*ZmBRZ1*, *ZmBRZ4*, *ZmBRZ5*, *ZmBRZ9*, ZmBZR10 and *ZmBRZ11*), as showed in Fig. 5B. *ZmBZR10* displayed the highest mRNA abundance in all tissues analysed. In the apical region of root it displayed values of expression 2/4-fold higher than those measured for *ZmBZR1*, *ZmBZR4*, *ZmBZR5*, *ZmBZR9* and *ZmBZR11* (section A), while in the maturation zone (section B), mRNA levels of *ZmBZR10* were 2/3-fold higher than the other. In stem region (section C) its expression was 6/10-fold higher than the others, while in leaves (section D) its transcripts were 2.5-fold more abundant than those of *ZmBZR9* and 3/4-fold higher than those detected for the other *ZmBZRs*. Except for *ZmBZR5*, which showed no significant differences in terms of spatial distribution of transcripts within plant, all the remaining genes displayed the highest amount of transcripts in stem.

Expression analysis under stress conditions

Figure 6 describes the change in transcript level measured in each of the four plant seedlings sections after stress treatments for the six *ZmBZR* genes selected, independently from their relative abundance. Under N starvation conditions, an increase in transcript accumulation of these genes was observed in the root tissues. In particular in the section A (root meristem enriched in transition and elongation zone) the amount of transcripts of *ZmBZR1*, *ZmBZR5* and *ZmBZR11* was 35/50% higher than those observed in control plants, *ZmBZR4* and *ZmBZR9* showed an increase of transcript level of 70/80% and *ZmBZR10* expression level was 2-fold higher respect to the control. In the stem and leaves (section C and D, respectively) the increase of transcript level was very low or insignificant for of all six ZmBZR genes. When seedlings were subjected to hypoxic stress, *ZmBZR11* was found to be the most responsive gene, with a 2-fold transcript increase in sections A and D, and a 2.5-fold increase in the section C compared with the control plants. A 2-fold transcript increase, limited to sections C and D, was also detected for *ZmBZR4* and, only in leaves, for *ZmBZR1*. An increase in the transcript level was registered also for the *ZmBZR5*, *ZmBZR9* and *ZmBZR10*, even though to a lower extent.

As also observed in the case of N deficiency, when plants were subjected to NaCl treatment roots were the most responsive tissues. In particular, *ZmBZR11* expression increased of 3-fold in section A and of 2-fold in section B, *ZmBZR9* expression increased of 2-fold both in section A and B. Furthermore, in this case the gene transcript accumulation was significantly induced also in leaves tissues for *ZmBZR1*, *ZmBZR4*, *ZmBZR5* and *ZmBZR10*, differently from what observed in the case of N starvation that affected only the level of transcript level in roots.

Finally, heat stress induced an opposite effect on gene expression by down-regulating ZmBZR transcript amount in nearly all tissues. In particular in root apex (section A), a transcript reduction of between 40/50% and of 60/80% was observed for *ZmBZR4*, *ZmBZ10*, *ZmBZR11* and for *ZmBZR1*, *ZmBZR5*, *ZmBZR9*, respectively. The same trend, even if less marked, was also observed in the root maturation zone, with a decrease of *ZmBZR10* and *ZmBZR9* transcription of 40% and 70%, respectively. Furthermore, *ZmBZR1* showed a strong down regulation (-60%) of its expression also in shoot.

Fig. 7 describes the transcript accumulation of all *ZmBZR* genes along the plant in response to various stress conditions. As mentioned before, in non-stressed maize seedlings the most abundant mRNA levels were generally detected in the stem region for all *ZmBZR* genes. However, in response to nitrogen starvation, the highest transcript amount was detected in the root apex for *ZmBZR1*, *ZmBZR4, ZmBZR9* and *ZmBZR10*, while *ZmBZR11* shows the same mRNA level in both root apex and stem. *ZmBZR5* did not evidenced significant differences in the transcript spatial distribution within plant. A similar pattern of expression was observed in salt-stressed seedlings, although with higher variability among genes. Indeed, *ZmBZR9* and *ZmBZR11* showed a 1.5/2-fold increase of the mRNA abundance in the root apex while, as far as the remaining *ZmBZR* genes are concerned, this pattern of induction was less pronounced. Regarding hypoxic stress, no evident re-localization of *ZmBZR1*, *ZmBZR5*, *ZmBZR9*, and *ZmBZR10* transcripts were showed. Conversely, O_2-deprivation induced an increase of the transcript level both of *ZmBZR4* and *ZmBZR11* in the stem. Finally, heat stress triggered a re-localization of *ZmBZR* genes in the shoot, except for *ZmBZR11*, for which no differences in terms of tissue distribution was observed.

Discussion

The identification of a new class of plant endogenous steroidal hormones, named brassinosteroids (BRs), is the result of decades of research. Nowadays, the role of BRs both in regulating multiple physiological functions and in responding to various biotic and abiotic stresses is well established (Nawaz et al., 2017). BR perception and signal transduction involve a signaling cascade that transduces the BR signal from the cell surface to transcriptional activation in the nucleus (Kir et al., 2015). BZR1 transcription factor plays a key role in the downstream BR signaling pathway, by activating thousands of genes and repressing similar number of genes including BR biosynthetic genes via a feedback loop (Zhu et al., 2013). Considering that BRs are unable to be transported long distance, it has been proposed that BZR1 transcription factors may also serve as major connecting points among other signaling pathways (Saini et al., 2015). To understand how BRs regulate plant growth and development, as well as, they act in responding to stress conditions, a wide characterization of the transcriptional networks through which BRs regulate gene expression is necessary. To this aim the identification of BZR1 family members would be essential to elucidate the BR transcriptional networks. However, most of the information about BR signaling has been obtained from the model dicot species *Arabidopsis thaliana*. Although many authors suggest that *Arabidopsis* BZR1 TFs might play a conserved role also in rice (Tong and Chu, 2012), specific components of this signaling pathway are far to be fully validated in maize.

In this work a systematic analysis was carried out to investigate the presence of BZR transcription factors in maize genome. A comprehensive set of 11 BZR transcription factors were identified and described from the current version (B73 RefGen_V3) of the B73 maize genome. In former publications, 6 and 15 BZR were identified in *Arabidopsis* and *Brassica*, respectively. BZR1 and its homologs represent a small family of plant specific proteins unrelated to any gene

outside the plant kingdom (Wang et al., 2002). The presence of several members that share a similarity of more than 80% may suggest that they have overlapping or redundant functions. Motif and domain scanning showed that all of the maize BZR have the conserved BZR motifs, indicating that these maize BZR have the typical structures of the BZR TFs. Dissection of the functional domains of BZR proteins has revealed highly conserved N-terminal domains that have DNA binding activity both *in vitro* and *in vivo* (Yin et al., 2005). The BZR1 DNA binding domain (encoded by the first exon) is the most conserved region of the BZR1 proteins, as reported by He and collaborators (He et al., 2005).

BZR1 and BES1/BZR2 transcription factors are unique to plants and share high similarity at the amino acid level (Wang et al., 2002). Although the overall amino acid sequence identity among ZmBZR1, AtBZR1 and OsBZR1 is low, higher sequence identity is found in domains of important function. However, the homology between the two Arabidopsis TFs BZR1 and BZR2 (88%) is much higher than that observed between each of them and ZmBZRs. These date confirm the hypothesis that BZRs resulted from gene duplication from BZR1 only after the separation of dicots and monocots during evolution (Bai et al., 2007).

Additionally, we investigated the organ-specific expression of 11 *ZmBZR* genes in 5-days maize seedlings. Five of them did not show any transcript accumulation at this stage of development. The remaining genes (*ZmBZR1*, *ZmBZR4*, *ZmBZR5*, *ZmBZR9*, *ZmBZR10* and *ZmBZR11*) were ubiquitously expressed in all the tissues examined, suggesting that they could function as growth regulators during maize development. In fact, recent studies have demonstrated that BR signaling pathway is required to regulate hypocotyl cell expansion (Gallego-Bortolome et al., 2012; Li et al., 2012; Oh et al., 2012), as well as, to promote the transition from meristematic cells to primordial cells in the shoot (Oh et al., 2011; Zhiponova et al., 2012). In the root apex, BRs are further involved in controlling root growth, both coordinating root meristem size and also root cell elongation (Fridman et al., 2014; Heyman et al., 2013; Vilarasa-Blasi et al., 2014; Vragovic et al., 2015). In all of these physiological processes BES1/BZR1 complex plays a pivotal role, interacting with several TFs in order to connect other signalling pathways. Interestingly, these observations could fits with our results, considering that both root apex and stem region registered the highest transcript accumulation in comparison with the other plant regions (i.e. root maturation zone and leaves) for most of the *ZmBZR* genes analysed in this work.

More importantly, in response to various stress conditions, the spatial transcript accumulation of all *ZmBZR* genes varied along the plant. This is not surprising considering than many studies have suggested essential roles for BRs in responding to various stresses; however, most of these results have been obtained by exogenously applied BRs, while the molecular basis of BR-mediated stress tolerance, including the involvement of BZR TFs, remain still elusive. Here, we demonstrate that all stress conditions tested cause a spatial transcript redistribution of BZR TFs throughout the young plant with respect to non-stressed conditions.

In response to N starvation, all six *ZmBZR* genes show an induction of their expression in the root system. The involvement of a BR signaling component in the regulation of the response to nutrients is to be expected, as, for example, phosphate deprivation reduces the expression of BR biosynthetic genes and shifts the intracellular localization of BZR1/BES1 (Singh et al., 2014); however, to date, it remains unclear how BR signaling is involved in N-stress responses. The application of exogenous brassinolide up-regulates a large number of *NRT* genes in *Arabidopsis* seedling roots grown on both high and low nitrate plates (Kiba et al., 2011). On the contrary, Trevisan et al. (2011) reported that the BR receptor-like kinase *BRI1* expression was down-regulated after 5 days of nitrate depletion in maize. Similarly, the BRI1 kinase inhibitor 1 gene *BKI1*, a negative regulator involved in the BR signaling pathway, was up-regulated under N deficiency in cucumber (Zhao et al., 2015). These apparently conflicting data might be explained considering that BRs perform diverse functions by sharing signaling pathways with other phytohormones. For example, it has been demonstrated that ABA inhibits plant growth by suppressing BR signaling downstream of BR receptor (Zhang et al.,

2009). An antagonistic interaction has been also evidenced between BRs and gibberellins, since the GA repressor DELLA directly interacts with BZR1 to inhibit its DNA binding and thus transcription activity in controlling photomorphogenesis (Sun et al., 2010; Li et al., 2012). In this scenario, given the apparent involvement of multiple phytohormones also in nitrogen signalling (Kiba et al., 2011), one future challenge will be to understand how BRs interact with other phytohormones to respond to N deficiency.

Regarding other abiotic stress conditions, such as hypoxia, salt and heat stress in plants, a large number of studies have demonstrated the ameliorating effect of exogenously applies BRs in promoting stress tolerance (Vardhini and Anjum, 2015). This positive action is generally correlated with higher expression of stress marker genes, indicating that increased expression of stress responsive genes is responsible, at least in part, for the higher stress tolerance in BR-treated plants (Vardhini and Anyum, 2015). In addition, it has been shown that application of BRs activates antioxidative pathways, including ROS-scavenging systems, as well as, non-enzymatic antioxidants, such as osmolytes like proline, glycine betaine, sorbitol, mannitol, and reduced glutathione, ascorbic acid that are needed for osmotic adjustment, stabilization of membranes, and ROS-scavenging (Fariduddin et al., 2014). However, it is still unclear whether BRs, directly or indirectly, modulate the responses of plants to oxidative stress. Interestingly, we found differential pattern of expression of all the *ZmBZR* genes in response to stress conditions. Most of these genes are highly up-regulated under both hypoxia and salt stress, suggesting that they might play a role in abiotic stress resistance in maize. Specifically *ZmBZR4* and *ZmBZR11* were found to be the most responsive gene under hypoxic conditions while *ZmBZR9* and, again, *ZmBZR11* were the most responsive to salt stress. An increase in the transcript level was also registered for the remaining genes, although, less pronounced. These data suggest that every single ZmBZR TFs may play a specific role in transducing different stress signals. Finally, it is worthy of attention the fact that heat stress clearly down-regulated gene expression of all *ZmBZRs* analysed. We speculate that this apparently contrasting result might be explained by considering the antagonistic interaction between BRs and ABA in regulating, for example, seed germination and dormancy during embryo maturation (Hu and Yu, 2014). More consistently with our results, it has been demonstrated that high endogenous levels of ABA suppresses BR-mediated responses in plant (Divi et al., 2010). In ABA deficient mutant *aba1-1* in fact, pronounced effects of exogenously BRs applied were observed under heat stress conditions due to higher accumulation of heat shock protein 90 (Divi et al., 2010). In this scenario, ABA conceals the effects of BRs in heat stress plant response and this interaction might involve the expression of BZR genes.

Consistently with the expression results, the analysis of the promoter regions of *ZmBZR* genes revealed the presence of a variety of cis-acting elements, regulating gene time and space expression levels. In addition to the hormone response elements, several stress and development-related elements were identified. The analysis revealed both a common and specific distribution of elements involved in different processes. These findings support the hypothesis that ZmBZR TFs play key roles in resistance to stress, defence against pathogen invasion, and the vegetative and reproductive growth of the plants.

In conclusion, these data reveal that BZR TFs have stress signaling activity in maize, in addition to their confirmed role in regulating plant physiology and morphology.

Author contributions

The work presented here was carried out in collaboration among all authors. AM and ST performed the experiments and wrote the manuscript. SQ conceived and designed the project, analysed data, wrote the manuscript and obtained

funds to support the project. SV contributed to concept the idea, helped in manuscript writing and obtained funds to support the project. All authors have read and approved the final manuscript.

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Figure captions

Fig. 1 Chromosomal locations of maize BZR TFs along ten chromosomes. Chromosome numbers (1 to 10) are indicated under each chromosome

Fig. 2 Schematic representation of motifs and intro-exon distribution identified in *Z. mays* L. BZR proteins. Different motifs are indicated by different colours, and the names of all members are shown on the left side of the figure, along with their phylogenetic relatedness. The intron–exon organization patterns of 11 ZmBZR TFs are shown in panel B, along with their intron splicing patterns. The amino acidic composition of each motif is reported in panel C

Fig. 3 Phylogenetic tree showing the relatedness of the deduced full-length amino acid sequences of 11 ZmBZR putative proteins and all BZR family proteins of *Arabidopsis*, rice, sorghum and wheat. ZmBZR proteins are shown in red

Fig. 4 (A) Number of each cis-acting element in the promoter region (1 kb upstream of ATG site) of *ZmBZR* genes. (B) The statistics of total number of *ZmBZR* genes including corresponding cis-acting elements (red dot) and total number of cis-acting elements in *ZmBZR1* gene family (blue box). Based on the functional annotation, the cis-acting elements were classified into three major classes: stress-, hormone-, development- and light responsiveness- related cis-acting elements. The regulatory elements and their descriptions are included in Supplementary Table S3

Fig. 5 (A) Schematic picture showing the division of maize seedling for sampling material for expression analyses. A and B represent two maize root zones. The section A is enriched in meristem, transition and the elongation zone. The mature zone of the root is named B. In C and D samples are included aerial parts (stem and leaves, respectively). (B) RT-qPCR validation of six *ZmBZR* genes (*ZmBZR1*, *ZmBZR4*, *ZmBZR5*, *ZmBZR9*, *ZmBZR10*, *ZmBZR11*) in four different plant portions. Seedlings were grown in a Hoagland-modified nutrient solution for 5 days. The levels of *ZmBZR* gene expression were measured in total mRNAs from: meristem-, transition- and elongation-enriched root zone (A), root maturation zone (B), stem (C) and leaves (D). Data were expressed as a.u., arbitrary units

Fig. 6 Heat map representation of RT-qPCR of differential relative expression of six *ZmBZR* genes in four plant sections (A, meristem-, transition- and elongation-enriched root zone; B, root maturation zone; C, stem; D, leaves). Analysis was conducted using two independent biological repetitions. The expression levels were normalized against the maize MEP gene. Data for each region are reported as stressed/non-stressed RT-qPCR relative expression values. The colour bar indicates high to low expression respect to the control

Fig. 7 Spatial distribution of six *ZmBZR* genes differentially expressed after stress treatments in different plant portions: meristem-, transition- and elongation-enriched root zone (A), root maturation zone (B), stem (C) and leaves (D). Transcript abundance (%) is recorded in non-stressed maize seedlings (column C) and in response to 5 days of nutritional (N), hypoxic (O), salt and heat (T°) stress. Percentages are expressed as the ratio between the mRNA abundance measured in each specific plant zone and the global amount of transcript in the overall seedling

Figure 1

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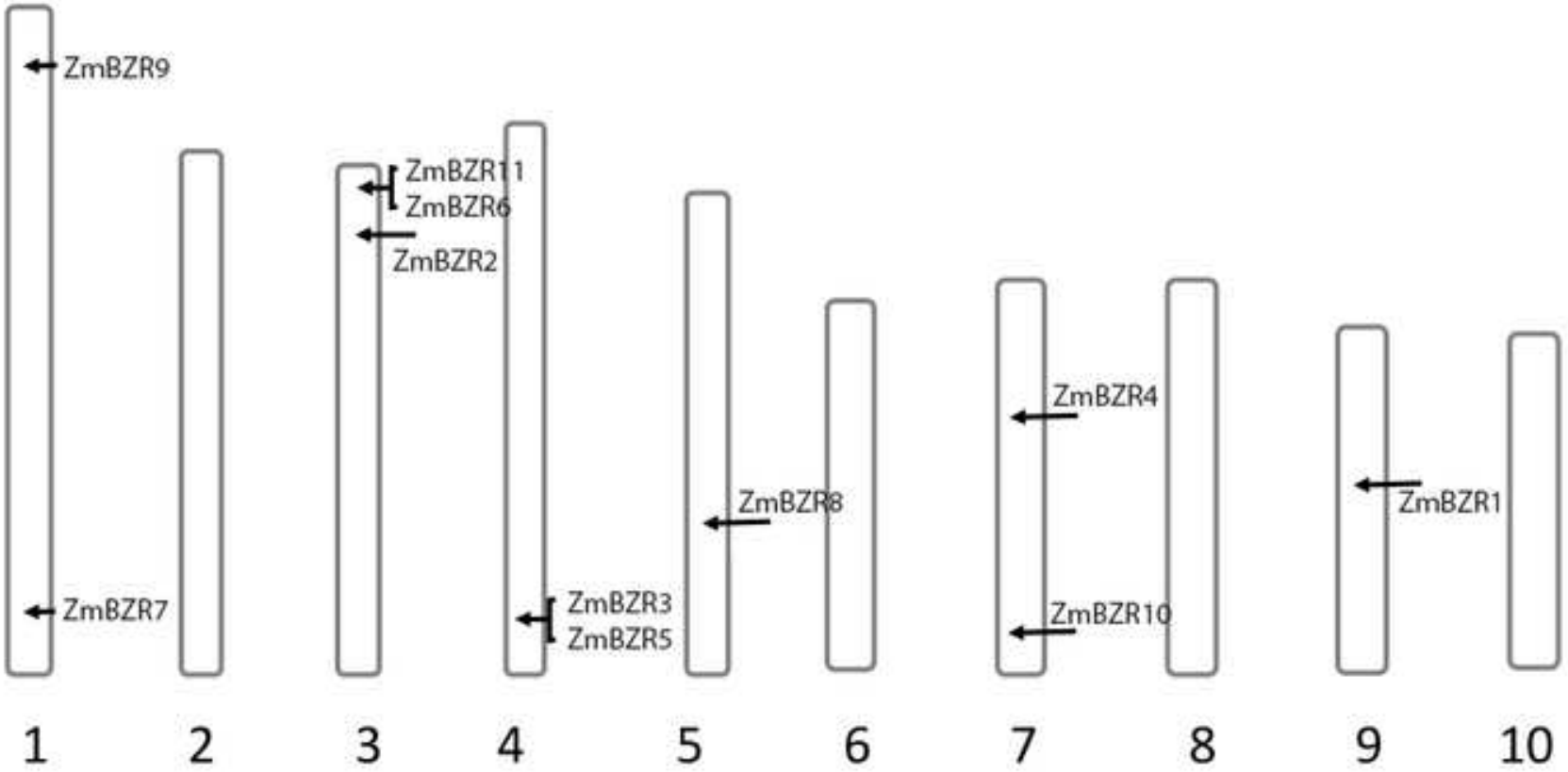

Figure 2

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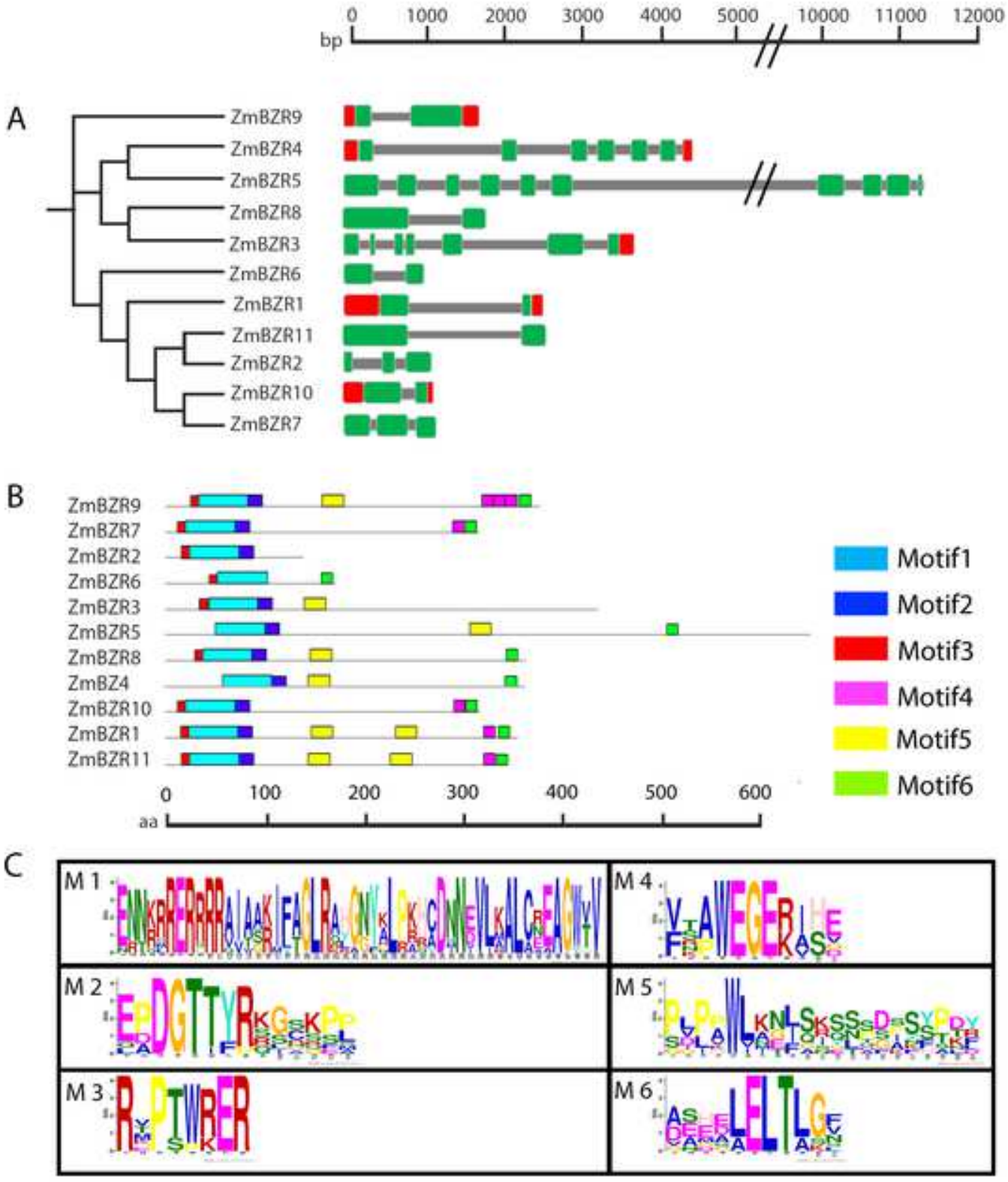

Figure

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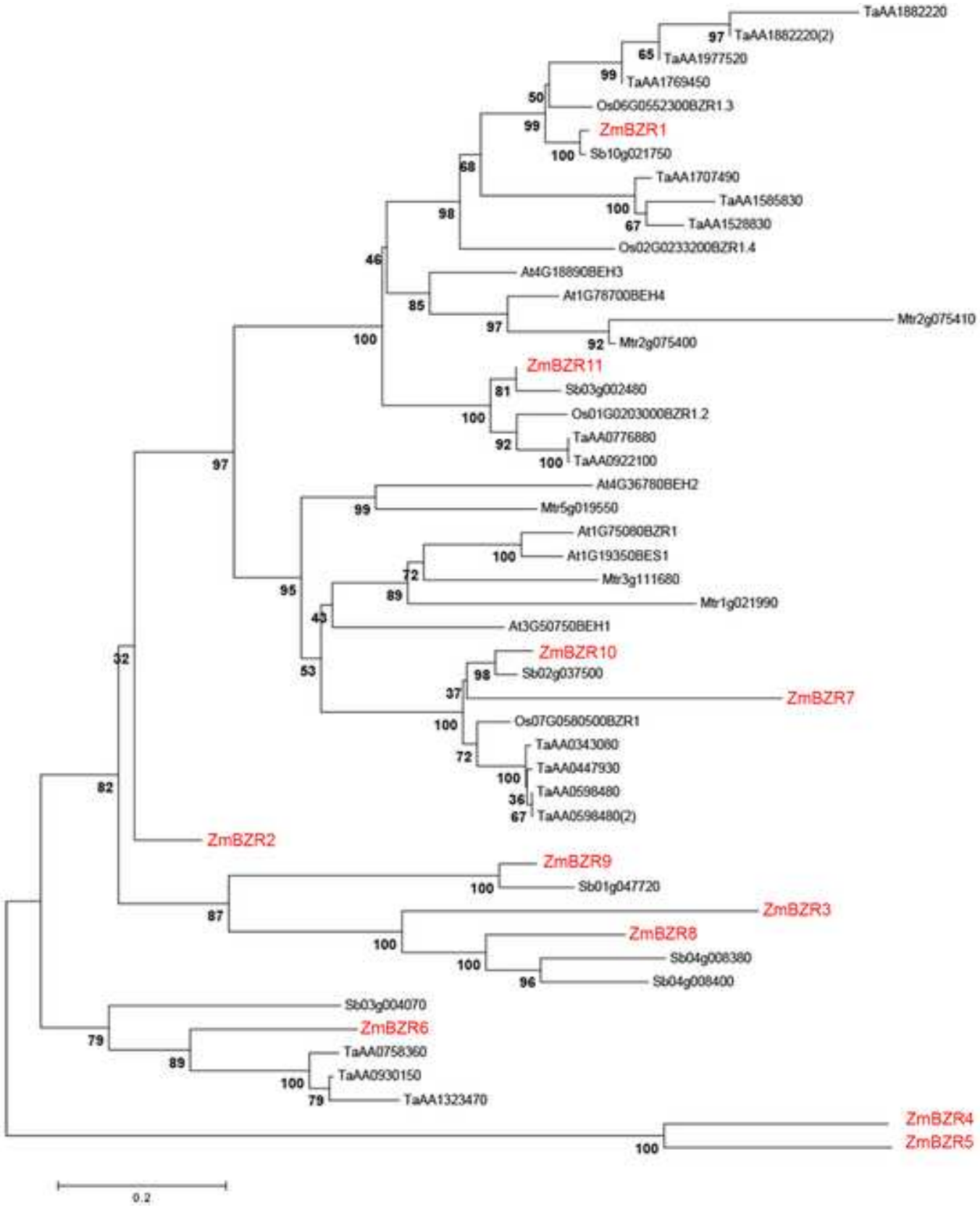

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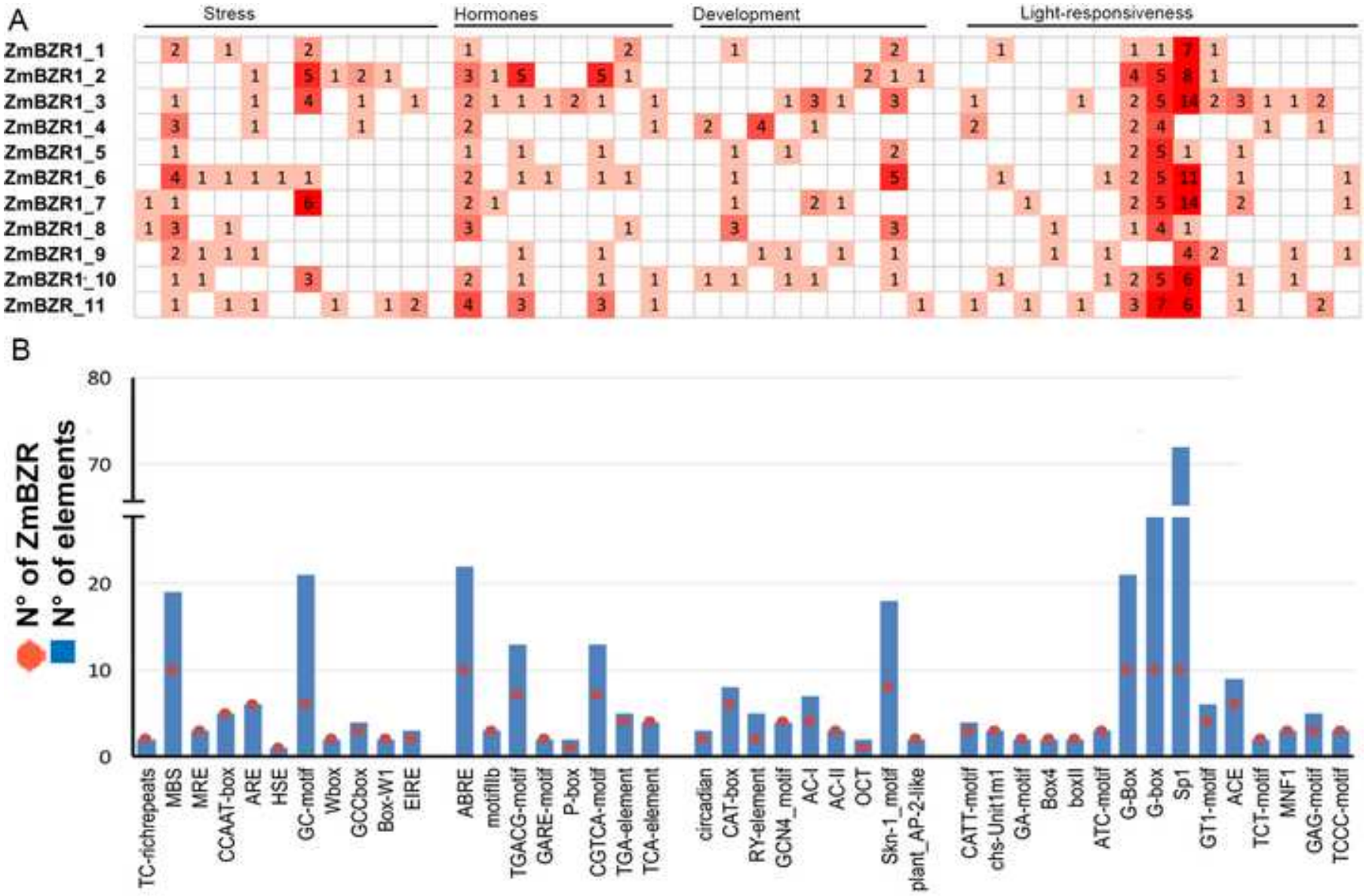

Figure 5

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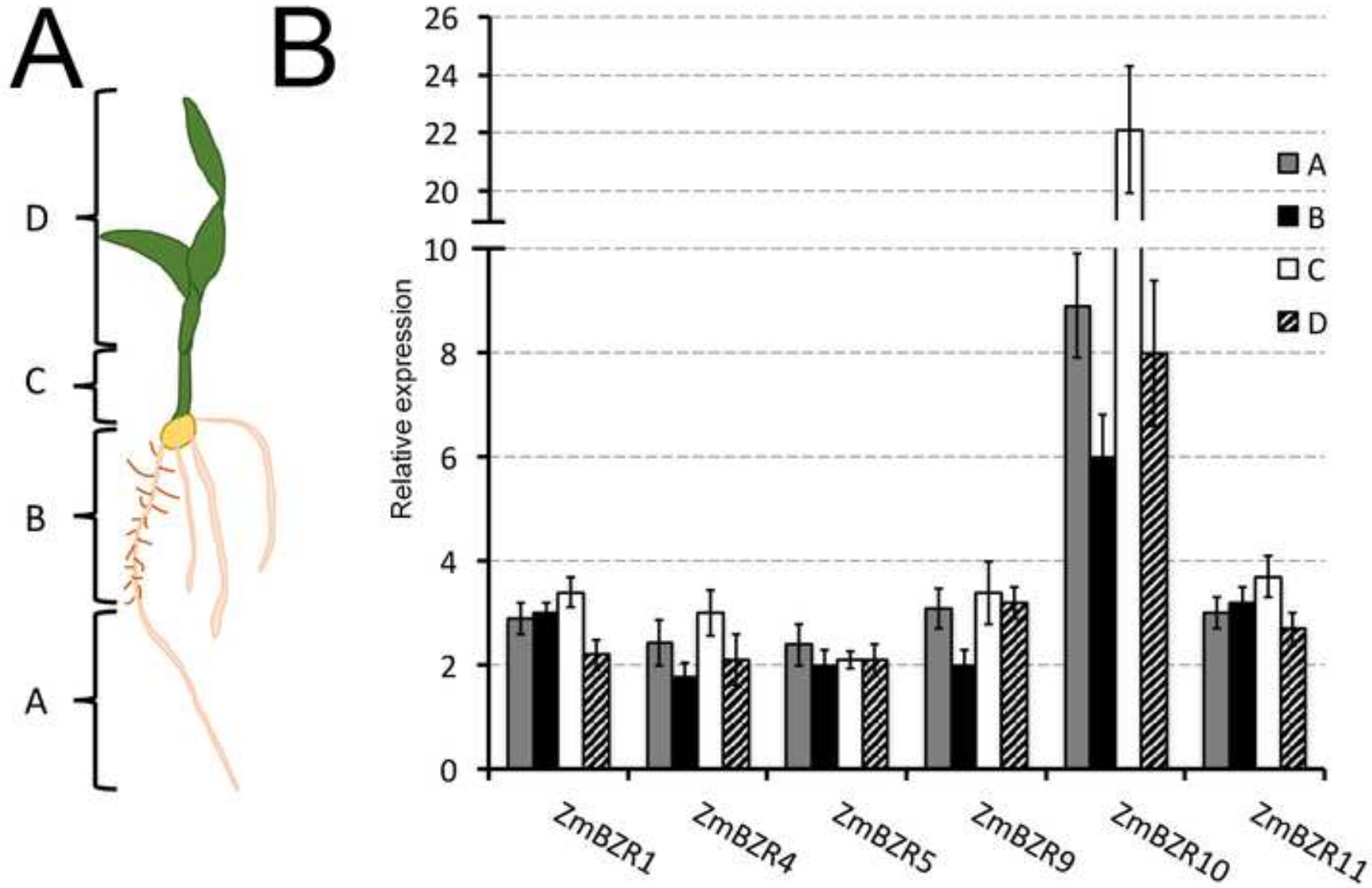

Figure 6

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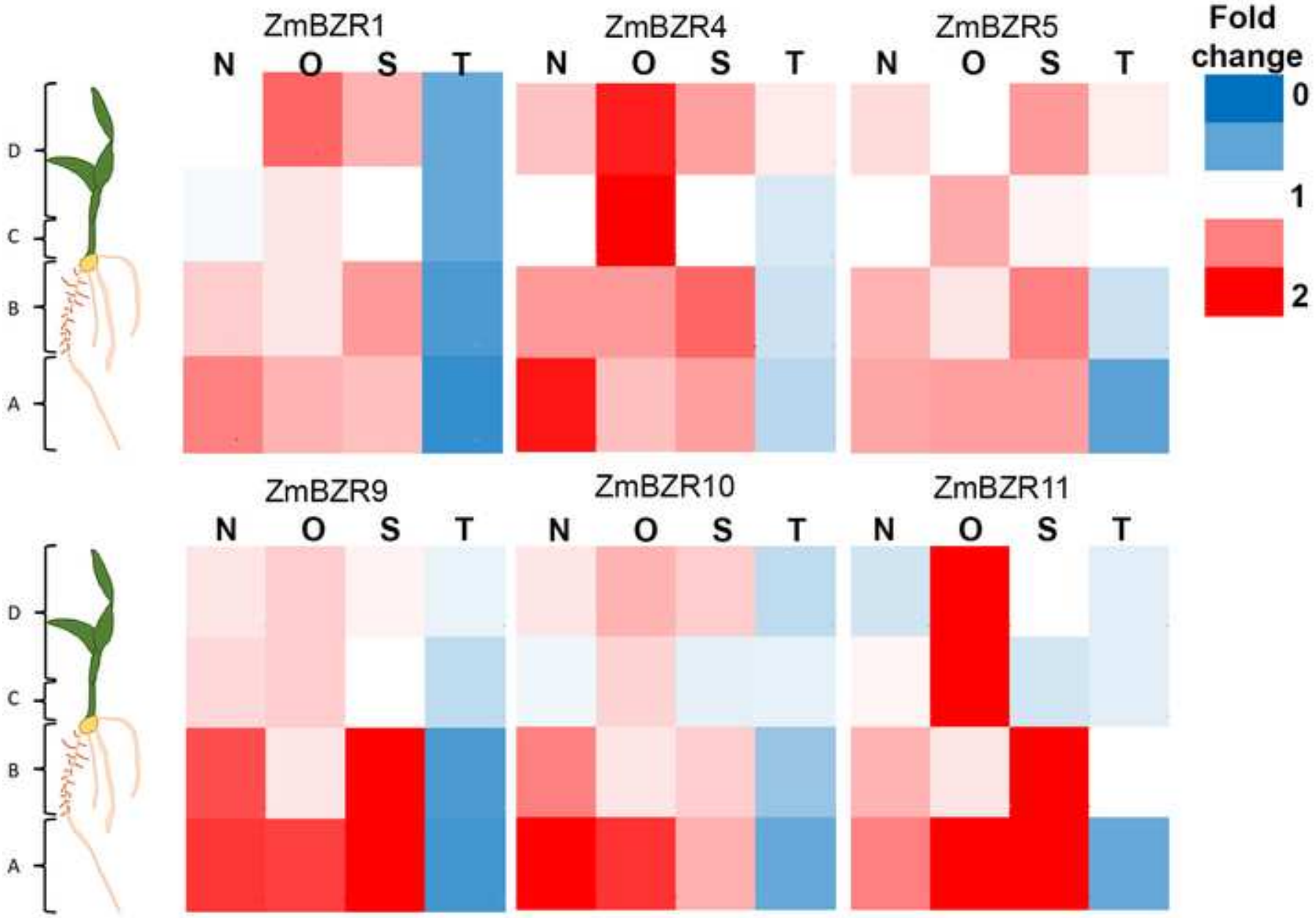

Figure

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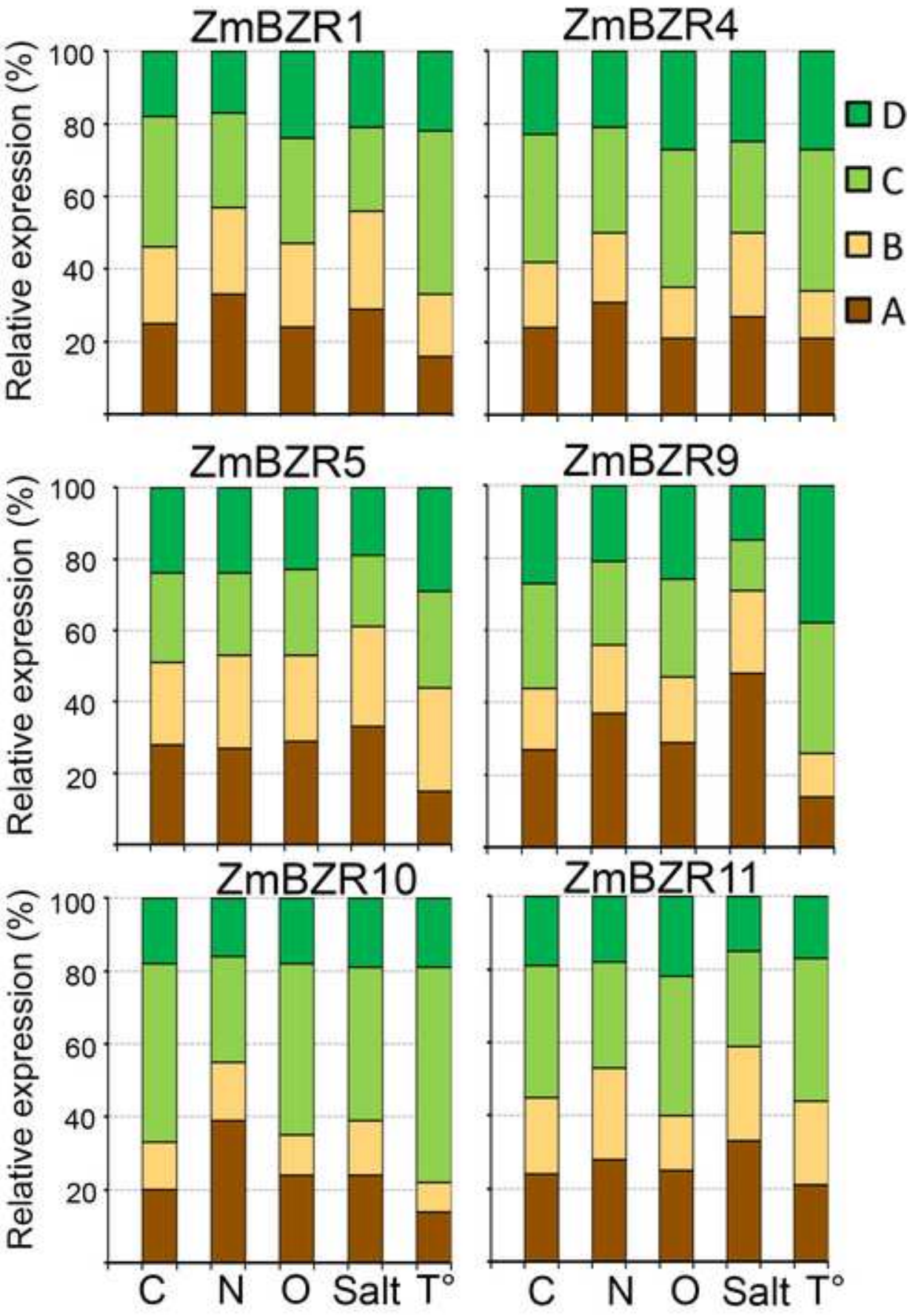

Table 1

In silico analysis of *BZR* genes collected from the *Zea mays* L. database (http://www.gramene.org/).

BZR code	Locus ID	Gene name	Chr.	ORF (bp)	Lenght (aa)	BZR domain start–end (aa)	iso-electric point	MW (g/mol)	No. of introns	Sub-cellular localization
BZR1	GRMZM5G812774	BES transcription factor; Brassinazole-resistant 1 protein	9	1891	355	13-157	8.0744	37414.3	1	plastid/nucleus
BZR2	GRMZM5G852801	Uncharacterized protein, BZR1, transcriptional repressor domain	3	420	139	15-83	10.7115	14,974.26	2	nucleus
BZR3	GRMZM2G307241	Uncharacterized protein, BZR1, transcriptional repressor domain	4	1311	436	34-168	9.4409	47979.89	8	nucleus
BZR4	GRMZM2G446515	Beta-amylase, BZR1, transcriptional repressor domain	7	2131	484	48-128	5.1148	54,940.34	6	plastid
BZR5	GRMZM2G069486	beta-amylase 2, BZR1, transcriptional repressor domain	4	2587	651	42-191	6.5986	73266.45	9	plastid/nucleus
BZR6	GRMZM5G868061	Uncharacterized protein, BZR1, transcriptional repressor domain	3	516	171	44-134	9.9655	18,755.71	1	nucleus
BZR7	AC194970.5_FG002	Uncharacterized protein, BZR1, transcriptional repressor domain	2	951	316	11-81	8.6982	33794.1	2	plastid/nucleus
BZR8	GRMZM2G369018	Uncharacterized protein, BZR1, transcriptional repressor domain	5	1095	363	29-118	10.7732	38163.42	1	nucleus
BZR9	GRMZM2G152172	Uncharacterized protein, BZR1, transcriptional repressor domain	1	1536	378	24-119	7.5381	38,904.48	1	nucleus
BZR10	GRMZM2G102514	BES1/BZR1	7	1478	317	11-154	8.6069	33,558.64	1	Nucleus
BZR11	GRMZM6G287292	Brassinazole-resistant 1 protein	3	1014	345	15-153	10.3186	27,005.47	1	nucleus

Supplemental Tables (S1-S2-S3)