This is an open access article distributed under the terms of the Creative Common Common BY-NC-ND Licence

Molecular characterization of the promoter of the stress-inducible *ZmMYB30* gene in maize

J. LUO, C.M. YU, M. YAN, and Y. H. CHEN*

School of Life Science, Nantong University, Jiangsu 226019, P.R. China

Abstract

The *ZmMYB30* is a member of the myeloblastosis (MYB) transcription factor superfamily, which has been shown to be a transcription regulator in abiotic stress tolerance in maize (*Zea mays*). To further identify the biological function of *ZmMYB30* and reveal how its expression is induced in response to stress, we isolated the *ZmMYB30* promoter and conducted a functional analysis. A 1461-bp promoter fragment was cloned and sequenced. Motif prediction using *PlantCARE* revealed several stress-responsive elements in the promoter sequence. Deletions in the promoter sequence affected the activity of the promoter and demonstrated that putative stress-responsive motifs, including TC-rich repeats (ATTCTCTAAC), abscisic acid responsive element (ABRE, ACGTG), and MYB binding site (MBS, CAACTG), played important roles in regulating the expression of *ZmMYB30*. Promoter β -glucuronidase (GUS) analysis also showed that *GUS* expression under the control of the *ZmMYB30* promoter responded to drought and salinity. Many natural variations in the *ZmMYB30* promoter sequence were found among 31 inbred maize lines, including 22 single nucleotide polymorphisms (SNPs), 17 insertion and deletion (InDels), and approximately 400-bp long deletions. The *ZmMYB30* expression varied greatly among the different inbred lines. The long fragment deletion in the promoter region did not impair the *ZmMYB30* expression, whereas SNP (-374) in the MBS motif in the 62R and LH82 lines greatly decreased the *ZmMYB30* expression. A site-specific mutation in the MBS motif also decreased the expression of the reporter *GUS* gene driven by the *ZmMYB30* promoter sequence. The expressions of three stress-responsive genes *ZmSOS1*, *ZmSOS2*, and *ZmABF3* were found to be consistent with the *ZmMYB30* expression. Our results provide new evidence to support the role of *ZmMYB30* as an important regulator in maize stress tolerance.

Additional key words: inbred maize line, MYB transcription factor, stress-responsive motif, stress tolerance.

Introduction

Maize is one of the most important crops all over the world. It provides essential raw material for food, forage, pharmaceuticals, and other industrial products. Environmental stresses, such as heat, cold, drought, and high salinity, frequently and drastically hamper plant growth and yield of maize worldwide. Higher plants, including maize, have evolved sophisticated genetic and molecular regulatory networks to respond and adapt to these stresses (Huang *et al.* 2012, Zhu 2016). A large number of studies have been conducted to better understand the stress tolerance mechanisms at physiological and

biochemical levels. Many genes involved in regulating abiotic stress responses have been identified in a variety of plants, including those that encode protein kinases, protein phosphatases, and transcription factor (TF) families, as well as functional downstream genes that encode ionic and osmotic balance maintainers (Abe *et al.* 2003, Yamaguchi-Shinozaki and Shinozaki 2006, Huang *et al.* 2012, Zhu 2016, Samad *et al.* 2017, Viana *et al.* 2018). Some of the identified genes have been used as genetic or molecular markers to genetically engineer crops with improved stress tolerance without sacrificing the yield and quality.

Among the various components of signaling pathways, TFs play pivotal roles in stress tolerance regulation

Submitted 13 September 2019, last revision 15 January 2020, accepted 20 January 2020.

Abbreviations: ABA - abscisic acid; ABF - ABRE-binding factor; ABRE - ABA-responsive element; AmTEA - *Agrobacterium tumefaciens*-mediated transient expression assay; CaMV - Cauliflower Mosaic Virus; D - deletion; FL - full length; GUS - β -glucuronidase; InDel - insertion and deletion; MBS - MYB binding site; MS - Murashige and Skoog; MYB - myeloblastosis; PEG - polyethylene glycol; qPCR - quantitative PCR; SOS - salt overly sensitive; SNP - single nucleotide polymorphism; TFs - transcription factors; WT - wild type.

Acknowledgments: This study was supported by grants from the Science and Technology Fund of Nantong City (MS12017022-7; JC2018158). We thank Margaret Biswas, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript. The first three authors contributed equally to this work.

* Corresponding author; e-mail: chenyh@ntu.edu.cn

because a single TF can orchestrate the expression of many genes to improve stress tolerance. Many experiments have shown that heterogeneous overexpression of a single TF-coding gene can increase the stress tolerance in transgenic lines (Chen *et al.* 2018, Zhao *et al.* 2018). Functional analysis of the stress-inducible TFs will help to classify the complex regulatory gene networks that trigger stress responses to drought, cold, and high salinity (Sun and Dinneny 2018). Functional analysis of specific TFs includes the identification of target genes regulated by the TFs and determination of how the gene expression and transactivation activity of TFs is controlled. The expression of target genes depends mainly on the interaction between *cis*-acting elements close to the gene sequence and TFs. Several conserved motifs have been verified in the regulatory regions of stress-inducible TF target genes, including abscisic acid (ABA)-responsive element (ABRE), and CRT/DRE, MYCRE, and MYBRE *cis*-elements, and the corresponding TFs that bind to them also have been identified (Guiltinan *et al.* 1990, Stockinger *et al.* 1997, Abe *et al.* 2003, Tran *et al.* 2004, Cheng *et al.* 2013, Xiang *et al.* 2017). The molecular mechanisms of some plant TFs involved in the regulation of downstream stress response genes have been well characterized, whereas how the TF genes are regulated remains to be fully elucidated. Some TF activity was shown to be regulated by signal transduction, such as phosphorylation and dephosphorylation, or by posttranscriptional modification (Tan *et al.* 2018, Zhou *et al.* 2019), and the expression of some TF genes was found to be regulated epigenetically by microRNAs or methylation (Roy 2015, Xu *et al.* 2015, Samad *et al.* 2017). Only a few studies have reported the regulation of gene expression by interactions between *cis*-elements and upstream TFs. For example, *ICE1* encodes a MYC-like bHLH TF that regulates the expression of the *CBF3/DREB1A* TF gene by binding to the ICER1 and ICER2 elements (Chinnusamy *et al.* 2003). Therefore, the interaction between upstream TFs and *cis*-acting elements in downstream target TF genes needs further study.

Maize is a model plant for functional genomic studies on TFs because of its abundant natural genetic variation, especially in the promoter regions of genes where *cis*-acting elements are located. In different inbred lines, certain single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) in the promoter regions often change the expression and patterns of the corresponding genes, which leads to the different phenotypes (Garg *et al.* 2012, Mao *et al.* 2015, Wang *et al.* 2016, Xiang *et al.* 2017). For example, three SNPs and two InDels upstream of the start codon in the maize DREB TF gene *ZmDREB2.7* were significantly associated with phenotypic variation in drought tolerance (Liu *et al.* 2013).

In recent years, MYB TFs, mainly in *Arabidopsis* but also in some crop species, have been characterized for their involvement in abiotic stress responses (Baldoni *et al.* 2015, Guo *et al.* 2018, Zhao *et al.* 2018). In a previous study, we identified a stress-responsive MYB gene in maize, *ZmMYB30* (GRMZM2G087955), and its ectopic overexpression in *Arabidopsis* increased the tolerance of the transgenic plants to salt stress (Chen *et al.* 2018). In

Arabidopsis, the orthologs of *ZmMYB30* (AtMYB44, AtMYB73, and AtMYB73) played multiple roles in ABA signaling and stress tolerance (Jaradat *et al.* 2013, Kim *et al.* 2013, Persak and Pitzschke 2013). To further reveal the biological function of *ZmMYB30* and the molecular mechanisms of *ZmMYB30* networks integrating multiple stress responses, we isolated a 1 461-bp promoter fragment of the stress-responsive *ZmMYB30* gene and a set of fragments with deletions in the promoter sequence from maize and analyzed their transcriptional regulation activity. Analysis of the regulatory region of *ZmMYB30* amongst 31 genotypes revealed many natural variations including many SNPs, small InDels, and > 400-bp long deletions. We also performed an association analysis of natural variations in *ZmMYB30* with their expression levels and stress tolerance activity in different inbred lines.

Materials and methods

Plants, growth conditions, and drought and salt stress treatment: The seeds of 31 maize lines (*Zea mays* L. cv. B73 and 30 inbred lines, see Fig. 1) were germinated on moistened gauze at 28 °C in the dark. The seedlings were then transferred to Hoagland's liquid medium and grown in a plant growth chamber (28 °C, 16-h photoperiod, 70 % humidity, 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance). Maize seedlings at the third leaf stage were treated with 150 mM NaCl for 12 h to mimic salinity stress.

Arabidopsis Col-0 seeds were surface sterilized and sown on Murashige and Skoog (MS) medium containing 1 % (m/v) sucrose and 0.8 % (m/v) agar. The plates were then placed vertically in a greenhouse (22 °C, 16-h photoperiod, 70 % humidity, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance) for 2 weeks. The 2-week *Arabidopsis* seedlings were used in an *Agrobacterium tumefaciens*-mediated transient expression assay (*AmTEA*) (Dhadi *et al.* 2012). After co-cultivation with *Agrobacterium* GV3101 for 20 h, the seedlings were exposed to dehydration and salinity stresses for 4, 8, and 12 h by substituting the MS medium with 20 % (m/v) polyethylene glycol 6000 (PEG6000) and 150 mM NaCl, respectively.

Cloning and putative *cis*-element analysis of the *ZmMYB30* promoter: Using the B73 maize genomic DNA as a template, the 1 461-bp promoter fragment was amplified by PCR using primer pairs FL-F and FL-R designed based on the full length (FL) reference B73 genome sequence (<https://www.maizegdb.org/>) (Fig. 2). The high fidelity enzyme Q5 (*New England Biolabs*, Ipswich, UK) was used in the PCR system and the PCR conditions were as follows: pre-denaturation at 94 °C for 3 min, then 33 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min 30 s, and final extension at 72 °C for 10 min. The PCR products were cloned into blunt vector pJET1.2/blunt (*Thermo Scientific*, Shanghai, China) and the positive clones were sequenced. Putative conserved *cis*-elements in the *ZmMYB30* promoter were analyzed using the *PlantCARE* database (Lescot *et al.* 2002).

Construction of expression vector and *Agrobacterium*-mediated transient transformation: To identify the functional regions and candidate stress-responsive *cis*-elements in the *ZmMYB30* promoter, the 1 461-bp FL promoter and a series of 5' deletion fragments of the *ZmMYB30* promoter named D1, D2, and D3 were amplified using the same R1 primer and different forward primers. A 486-bp deletion fragment at the 3' of promoter R4 (amplified by R4-R and FL-F) and positive control 35S promoter fragment also were obtained. A mutation site, named M, was introduced into the stress-responsive MBS site by overlapping PCR (Urban *et al.* 1997). All the primers are listed in Table 1 Suppl., and primer positions are indicated in Fig. 2. The promoter fragments were cloned into the pBII01.1 vector through the *Hind*III and *Bam*HI sites to drive the expression of the β -glucuronidase (*GUS*) reporter gene. The recombinant constructs were confirmed by sequencing and then transformed into the *A. tumefaciens* GV3101 strain using the freeze-thaw method. The transient transformation experiments were carried out using the *AmTEA* protocol (Dhadi *et al.* 2012). For co-cultivation procedures, 10 cm³ of the overnight grown in Lurie and Bertani (LB) broth *Agrobacterium* GV3101 cultures harbouring different constructs of interest (absorbance approximately 1) were diluted with 30 cm³ of LB broth. Ten *Arabidopsis* seedlings were co-cultivated with the diluted *Agrobacterium* GV3101 supplemented with 100 μ M acetosyringone (20 mm³, *Sigma-Aldrich*, St. Louis, USA) and *Silwet L-77* (10 mm³, *Lehle Seeds*, Round Rock, TX, USA) at 28 °C for 20 h. To prevent bacterial contamination, the seedlings were washed three times with sterile distilled water or 0.5 MS salt solution supplemented with 500 mg dm⁻³ carbenicillin after co-cultivation. Then, the plants were placed in a sterile 15-mm Petri dish and incubated for 12 h with 15 - 20 cm³ of MS salt solution supplemented with carbenicillin (500 mg dm⁻³). The *AmTEA* experiments were repeated three times for *GUS* signal staining and RT-qPCR analysis.

Histochemical staining *GUS* was carried out as described by Chen *et al.* (2007). Briefly, after the *AmTEA* experiments, the *Arabidopsis* seedlings were immersed in *GUS* staining buffer [1 mg cm⁻³ *X-Gluc*, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 10 mM EDTA, 0.1 % (m/v) *Triton X-100*, and 100 mg cm⁻³ chloramphenicol in 50 mM sodium phosphate buffer, pH 7.0] at 37 °C for 24 h. The chlorophyll of the stained seedlings was removed by incubating in 70 % (v/v) ethanol. The stained samples were photographed using a *Leica V-lux4* camera (*Leica Microsystems*, Wetzlar, Germany).

Extraction of DNA and RNA, and real time quantitative PCR analysis: The genomic DNA was extracted from the maize seedlings using a plant genomic DNA extraction kit (*Tiangen*, Beijing, China) according to manufacturer's instruction. Total RNA was extracted from the maize and *Arabidopsis* seedlings using a *TaKaRa MiniBEST* plant RNA extraction kit (*TaKaRa*, Dalian, China). DNA contamination was removed by digesting

the samples with RNase-free DNase I (*TaKaRa*) on a column. For cDNA synthesis, 3 μ g total RNA from the different seedlings was used for reverse transcription with a *PrimeScript*TM RT reagent kit with gDNA eraser (*Perfect Real Time*) (*TaKaRa*) according to the manufacturer's recommendations. For qPCR, 20 mm³ reaction systems containing 10 mm³ of 2 $\times$ *SYBR Green PCR Master Mix* (*Roche*, Basel, Switzerland), 2.0 mm³ of cDNA (10-fold dilution), and 1 mm³ of gene-specific primers (2.0 μ M) were set up. The amplification conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and at 60 °C for 30 s. The melting curve of each product was obtained to verify the specific amplification. The actin genes *Actin2* (AT3G18780) from *Arabidopsis* and *Actin8* (GRMZM2G126190) from maize were used as reference genes for the *Arabidopsis* and maize genes qPCR analyses, respectively. The PCRs were performed on an *ABI Prism 7000* system (*Applied Biosystems*, Waltham, USA). The reactions were repeated three times, with three biological replicates each. The results were analyzed quantitatively using the 2^{- $\Delta\Delta C_t$} method. The gene-specific primers for *ZmMYB30*, *GUS*, and the maize stress-responsive genes are listed in Table 1 Suppl.

Variation of *ZmMYB30* promoter sequence and association analysis with salinity tolerance: The *ZmMYB30* promoter sequences from different inbred maize lines, including the reference *ZmMYB30* promoter sequence (<http://www.maizegdb.org/>) from B73, were amplified using primer pairs FL-F + R1 (Table 1 Suppl.). All the obtained promoter sequences were cloned into a pJET1.2/blunt vector and sequenced. The *BLAST* on the *NCBI* website was used to identify nucleotide polymorphisms, including SNPs and InDels (Zhang *et al.* 2000). The correlation between the polymorphisms and the *ZmMYB30* expressions in the inbred lines was determined by qPCR. The expression patterns of three other maize stress-responsive genes also were detected in representative inbred lines.

Results

To identify the putative *cis*-acting elements in the *ZmMYB30* promoter sequence in the B73 inbred line, a 1 461-bp sequence upstream of the 5'-UTR was obtained by PCR amplification and cloned. The promoter fragment was sequenced and was found to share 100 % sequence identity with the B73 reference genome sequence. Bioinformatics analysis of the promoter sequence using *PlantCARE* revealed putative *cis*-acting regulatory elements that have been found to modulate gene expression in various species (Fig. 2) (Lescot *et al.* 2002). Besides the common core promoter elements (CAAT-box, TATA-box), several stress responsive elements, including ABRE (ACGTG), MBS (CAACTG), and TC-rich repeats (ATTCTCTAAC), were found. The detected putative *cis*-acting elements and their positions in the promoter sequence are listed in Table 1.

To fully identify the DNA polymorphisms present in the *ZmMYB30* promoter, 31 inbred maize lines were

Table 1. Putative *cis*-acting elements and their positions in the *ZmMYB30* promoter.

	Sequence	Position	Function
TATA box	ATATAA	-35, -124	core promoter element around -30 of transcription start
CAAT-box	CAAAT	-58, -156, -214, -1189	common <i>cis</i> -acting element in promoter and enhancer regions
<i>Cis</i> element	CGTCA	-1275	<i>cis</i> -acting regulatory element involved in MeJA-responsiveness
TC-rich repeats	ATTCTCTAAC	-981(-)	<i>Cis</i> -acting element involved in defense and stress responsiveness
I-box	GATAAGGTG	-1119	part of a light responsive element
ARE	AAACCA	-1264, -1107, -926, -1001 (-)	<i>cis</i> -acting regulatory element essential for the anaerobic induction
ABRE	ACGTG	-677, -511(-)	<i>cis</i> -acting element involved in the abscisic acid responsiveness
CCAAT-box	CAACGG	-388	MYBHv1 binding site
MBS	CAACTG	-372(-), +117	MYB binding site involved in drought-inducibility
TGA-element	AACGAC	-262, -705	auxin-responsive element
AT-rich sequence	AT-rich sequence	-930	element for maximal elicitor-mediated activation

Table 2. Variation of DNA sequence in the promoter region of *ZmMYB30* among 31 inbred lines. SNP - single nucleotide polymorphism, InDels - insertion and deletion.

DNA site	SNPs type	variants frequencies	DNA site	InDels type	variant frequencies
-1134	G→A	3/31	-1283	+1(A)	1/31
-714	C→T	4/31	-1250	-1(T)	5/31
-701	T→A	4/31	-1245	-1(T)	2/31
-698	A→G	4/31	-1244	+1(A)	3/31
-697	A→G	4/31	-1243	-1(A)	2/31
-667	T→C	5/31	-1033	+1(A)	2/31
-653	C→A	4/31	-946	+1(T)	1/31
-556	T→A	4/31	-900	-1(A)	18/31
-555	A→G	4/31	-856	+1(C)	1/31
-474	C→T	3/31	-615	+1(A)	6/31
-462	A→T	3/31	-557	-1(T)	4/31
-432	T→C	1/31(NC250)	-551	+1(T)	3/31
-407	G→T	10/31	-549	+2(TG)	1/31
-388	A→G	1/31	-505	+1(C)	1/31
-379	T→C	13/31	-475	+1(C)	1/31
-374	C→G	3/31 (62R, LH92, LH82)	-461	+1(T)	3/31
-361	T→G	13/31	-313-+119	-428	10/31
-344	T→G	3/31	-313-+96	-405	3/31
-208	T→C	1/17	+9	+1(AA)	3/18
-203	T→C	1/31			
+124	G→A	10/31			
+140	C→T	10/31			

selected randomly (Fig. 1). A 1 461-kb fragment spanning the *ZmMYB30* promoter region was cloned and sequenced from the 31 selected lines. The 31 sequences were aligned using the *NCBI's BLAST* server and the results were visualized using the *NCBI* alignment viewer (Fig. 1). The sequences of four inbred lines, PHR32, 191s, 71S, and 61S, were identical to the B73 reference genomic sequence. The other promoter sequences had many variations compared with the reference sequence. In total, 22 SNPs and 19 InDels were discovered, and the variation DNA sites, variation type, and variation frequencies are listed in Table 2. Prominent variations were 428-bp and 405-bp long

deletions in 10 and 3 inbred lines, respectively (Fig. 2, Table 2). Among the 22 SNPs, only 2 were located in the putative *cis*-element sequences, namely SNP (-388) and SNP (-374), which were in the MYBHv1 and MBS sites, respectively (Fig. 2). SNP (-379), SNP (-361), and InDel (-900) were correlated with the 428 bp and 405 bp long deletions. Similarly, SNPs (-407), (+124), SNP (+140) were correlated with the 428 bp long deletion, and SNPs (-374), (-474), (-462), and (+344), and InDels (-1244) and (+461) were correlated with the 405-bp long deletion. The correlation between different SNPs and deletions means that these variations were present simultaneously in the

promoter regions of some inbred lines.

Using the *AmTEA* protocol, the FL::GUS construct was transiently transformed into *Arabidopsis* seedlings to monitor the expression of *GUS*. The *CaMV35S* promoter, which was cloned into the upstream region of *GUS* was used as a positive control (Fig. 3A). The *GUS* gene expression was analyzed by plant tissue *GUS* histochemistry staining and RT-qPCR. The 1461 bp long fragment had transcription activity and drove the expression of *GUS* (Fig. 3B,C). The 150 mM NaCl and 20 % PEG 6000 treatments that mimic salinity and drought stresses enhanced the expression of *GUS* (Fig. 3D). At 4 h after the 150 mM NaCl treatment, the *GUS* expression reached a peak and then decreased. After the 20 % PEG 6000 treatment, the *GUS* expression increased at 4 h, then decreased at 8 h, and finally reached a peak at 12 h (Fig. 3D).

To further study the functions of the putative motifs in the gene promoter, three 5'-end deletion promoter::GUS vectors were constructed, and then transferred into *Arabidopsis* seedlings for transient expression analysis. The deletion promoters were named D1 (5'-end 447-bp deletion of FL), D2 (5'-end 635-bp deletion of FL), and D3 (5'-end 981-bp deletion of FL) (Fig. 3A). In the variation analysis of the different natural inbred lines, a long deletion of > 400 bp was found in 13 lines, so it was important to obtain a construct with a deletion that imitated this natural variation, that construct was named D4 (3'-end 486-bp deletion of FL) (Fig. 3A). The FL, 35S positive control, and D1 - 4 four deletion constructs were transiently transformed into *Arabidopsis* seedling to demonstrate the transcription activity of the different deletion promoters. The GUS staining detected GUS

enzyme signals in the *Arabidopsis* seedlings transformed with the 35S promoter positive control, and the FL, D1, and D2 constructs, whereas no GUS enzyme signals were detected in the *Arabidopsis* seedlings transformed with the D3 and D4 constructs (Fig. 3B). In the GUS staining experiments, apart from the positive control 35S promoter, which all parts organ of all seedlings were blue stained, while in other constructs, the blue stain signals appeared unevenly, only some seedlings and some organs of seedlings were stained. That's might attributed to the *Agrobacterium* infiltration or GUS staining procedure. It is hard to quantify the expression level and only we can figure out the presence or absence of the signal. So we detected the mRNA level of GUS by RT-qPCR and to quantify the expression level.

The *GUS* transcript was detected in all the constructs, including D3 and D4 (Fig. 3C). All the deletion constructs had lower *GUS* transcript expression than the FL construct. The D3 expression was very low, whereas the D4 expression was higher than the D1 and D2 expressions, which contradicts the GUS enzyme staining results (Fig. 3C,B). It may be that the deletion in D4 did not impair transcription of *GUS* but inhibited its translation, because the 3'-end 486-bp deletion of FL in the D4 construct may have removed at least part of the sequence necessary for translation initiation.

Through overlapping PCRs, we made base substitution in the conserved motif MBS (CAACTG) to verify its function. The mutated sequence was cloned into the upstream region of *GUS*. The results showed that the mutation caused a large decrease in the expression level of *GUS* (Fig. 3C).

Fig. 1. Sequence alignment of 31 promoter sequences from 31 inbred lines. Promoter sequences from B73 and other 30 inbred lines were aligned with the *BLAST* tool (align two or two more sequences) from the *NCBI* website. The alignment results were observed through the *NCBI* alignment viewer, and the graph was drawn in the *PhotoShop* software. The deletion region is indicated with a *dashed line*. According to the long deletion, 31 inbred lines were subdivided into 2 groups, a full length group and a long deletion group. The putative *cis*-elements and other natural variations (single nucleotide polymorphisms and small insertions and deletions) were omitted in this graph.

Fig. 2. The sequence of DNA and putative *cis*-acting elements in *ZmMYB 30* promoter predicted by the *PlantCARE* database. Putative *cis*-acting elements and consensus sequences detected in the promoter fragment using the *PlantCARE* database are indicated with grey shaded boxes (a positive strand) and underlined (a negative strand). The predicted transcription initiation site is indicated with +1. Eight primers (FL-F, D1-F, D2-F, D3-F, D4-R, FL-R, M-F, and M-R) for promoter::GUS constructions are marked on the promoter sequence. The position of two single nucleotide polymorphisms (SNPs, -374 C→G, -432 T→C) and > 400 bp deletion are labeled on the sequence. Insertion and deletion (InDel) 428, 405S indicates the start point of deletion, InDel 428 T, 405T indicates the terminator point of InDel 428 and InDel 405, respectively. The diamond indicates the mutation base pairs in M-F and M-R primers.

Fig. 3. Promoter activity analysis. The full length promoter sequence and a series of promoter deletions were cloned into pBI101.1 through *Hind*III and *Bam*HI to monitor the expression of the β -glucuronidase reporter gene. Different constructs were transiently transformed into *Arabidopsis* to detect the *GUS* expression under normal and stress conditions by *GUS* staining and reverse transcription quantitative PCR (RT-qPCR) analysis. **A** - A schematic representation of the *PZmMYB30::GUS* construct in the pBI101 vector. The insertion position of the *ZmMYB30* promoter in the vector is indicated with restriction enzyme sites (*Hind*III and *Bam*HI). LB - left border; RB - right border; P NOS - nopaline synthase promoter; NOS-ter - nopaline synthase terminator; P CaMV 35S - cauliflower mosaic virus 35S promoter, GUS - β -glucuronidase gene; NPTII - neomycin phosphotransferase (II) coding region; P ZmMYB30FL - *ZmMYB30* gene 1461bp promoter. D1 to D4 - promoter deletion fragments. Five kinds of shapes indicate different putative elements, respectively. **B** - Histochemical staining *GUS* activity in two-week-old *Arabidopsis* seedlings, which were transformed transiently with different promoter::GUS constructs described in **A**. **C** - RT-qPCR analysis of *GUS* expression in two-week-old *Arabidopsis* seedlings, which were transformed transiently with different promoter::GUS constructs described in **A**. The *Arabidopsis Actin2* gene was used as a reference gene. The minimal promoter construct D3 was referred as controls (B73) and set to 1. Error bars indicate standard deviations of triplicate experiments. *, ** - significant differences between control and detected samples, $0.01 < P < 0.05$ and $P < 0.01$, respectively, the Student's *t*-test. **D** - Two-week-old *Arabidopsis* seedlings were transformed transiently with the P ZmMYB30FL construct and treated with salinity and drought stresses, the time course of the *ZmMYB30* transcriptions after treatments was detected by RT-qPCR analysis. NaCl-0,4,8,12 indicates the time after 150 mM NaCl treatment; PEG-0,4,8,12 indicates the time after 20 % (m/v) PEG treatment. The *Arabidopsis actin2* gene was used as a reference gene. The *Arabidopsis* seedlings without treatment were referred as controls (NaCl-0, PEG-0) and set to 1. Error bars indicate standard deviations of triplicate experiments. *, ** - significant differences between control and detected samples $0.01 < P < 0.05$ and $P < 0.01$, respectively, the Student's *t*-test).

Fig. 4. The expressions of *ZmMYB30* and three stress responsive genes in maize inbred lines. *A* - The expression of *ZmMYB30* under normal conditions and NaCl stress detected by reverse transcription quantitative PCR in 12 inbred lines. Six non-deletion lines (B73, PHR32, LH190, Q381, 33-56, and 2FACC) and six deletion lines (NC250, MBST, LH193, H99, LH82, and 62R) were selected as representatives for two groups. The B73 and B73N, for example, indicate the expression under normal conditions and NaCl stress, respectively. The maize *actin8* gene was used as a reference gene. The B73 maize seedlings without treatment were referred as controls (B73) and set to 1. Error bars indicate standard deviations of triplicate experiments. *, ** - significant differences between samples treated with and without NaCl (e.g. B73 and B73N); a, b - significant differences between B73 and other samples and significant differences between B73 and other samples both treated with NaCl ($0.01 < P < 0.05$ and $P < 0.01$, respectively, the Student's *t*-test). *B* - The expressions of three stress related genes (*ABF3*, *SOS1*, and *SOS2*) under normal conditions and NaCl stress were detected by reverse transcription PCR in six inbred lines. The B73 and B73N, for example, indicate the expression under normal conditions and NaCl stress, respectively. The maize *actin8* gene was used as a reference gene. The B73 maize seedlings without treatment were referred as controls (B73) and set to 1. Error bars indicate standard deviations of triplicate experiments. *, ** - significant differences between samples treated with and without NaCl; a, b - significant differences between B73 and other samples and significant differences between B73 and other samples both treated with NaCl ($0.01 < P < 0.05$ and $P < 0.01$, respectively, the Student's *t*-test).

Differences among the sequence polymorphisms detected in the *ZmMYB30* promoter sequence from 31 inbred maize lines caused us to subdivide them into two groups, a long deletion group (13 inbred lines) and a full length group (18 inbred lines). To study the correlations between the *ZmMYB30* expression and the long deletion (428 bp/405 bp) and other variations in the promoter region, the expressions of the *ZmMYB30* transcripts were determined in the different inbred lines after they were treated with or without NaCl by RT-qPCR. We selected six lines from the full length group (B73, PHR32, LH190, Q381, 33-56, 2FACC) and six lines from the long deletion group (NC250, MBST, LH193, H99, LH82, 62R) as representatives of the two different groups. The results showed that the long deletion in the promoter region did not block the transcription of *ZmMYB30* (Fig. 4A). Three of the long deletion lines (NC250, 62R, LH82) had very low expressions compared with the other three long deletion lines (MBST, H99, LH193) (Fig. 4A). Notably, MBST and H99 had higher expression than B73 and other full length lines when cultured under normal conditions (Fig. 4A). Under the salt stress condition, the *ZmMYB30* expression was upregulated in all detected lines (Fig. 4A). The increase in expression was more obvious in B73 (Fig. 4A). SNP (-374), a C→G transversion, was present in the MBS motifs in 62R and LH82, which may explain the extremely low expressions in these two lines, SNP (-432), a T→C transition, is a unique natural variation in NC250.

To study the salt stress-responsive activity in the different inbred lines, we selected three maize salt stress-responsive genes, *ZmSOS1*, *ZmSOS2*, and *ZmABF3*, and monitored their expression patterns in B73, 62R, NC250, H99, LH190, and PHR32 treated with and without NaCl. The RT-qPCR results showed that the expressions of these three genes co-regulated with the *ZmMYB30* expression (Fig. 4B). In B73, H99, and LH190, the salt treatment increased the expression of *ZmMYB30* as well as the three salt-responsive genes (Fig. 4B). In PHR32, the salt treatment increased the expression of *ZmMYB30* but not the expressions of the three salt-responsive genes. In 62R and NC250, the low expression of *ZmMYB30* was consistent with the low expressions of the three salt-responsive genes (Fig. 4B).

Discussion

The *PlantCARE* analysis revealed several putative stress-responsive elements including ABRE (ACGTG), MBS (CAACTG), and TC-rich repeats (ATTCTCTAAC) in the *ZmMYB30* promoter. ABRE is a major *cis*-acting element in ABA-responsive gene expression. It mediates the ABA-dependent stress response in salt and drought stress tolerance. Analysis of the deletion promoter D1, D2, and D3 constructs showed that deletion of one, two, or three *cis*-elements dramatically decreased *GUS* expression. The drought and salinity stress treatments induced the expression of the *GUS* reporter gene driven by the *ZmMYB30* promoter (Fig. 3D), which mimics the expression pattern of *ZmMYB30* in the B73 inbred

line (Fig. 4A). The induced expression of *ZmMYB30* by salinity stress also was found in the six inbred lines tested (Fig. 4A).

We found many natural variations in the *ZmMYB30* promoter sequence among 31 inbred maize lines. Besides SNPs and small InDels, long fragment deletions (> 400 bp) were found in 13 of the 31 lines. To uncover the function of this fragment in regulating *ZmMYB30* expression, we conducted a promoter deletion analysis using *GUS* as the reporter gene. The promoter deletion analysis showed that the *GUS* expression from the 3'-end deletion D4 construct was similar to the expressions from the 5'-end deletion D1 and D2 constructs (Fig. 3C). In inbred lines the expressions of *ZmMYB30* were not affected by the 3'-end long deletions (Fig. 4A). Further, the deletions did not affect the stress-induced expression pattern of *ZmMYB30* (Fig. 4A). These experiments suggested that the *cis*-elements in the 3'-end of the *ZmMYB30* promoter were not important for regulating the expression of *ZmMYB30*. The expression of the *GUS* reporter gene driven by the 550-bp long promoter fragment (D3 construct) was very low. Other SNPs and InDels that affected *ZmMYB30* expression were SNP (-374) in the MBS *cis*-element and SNP (-432). SNP (-374) changed the putative MBS *cis*-element in three inbred lines, (62R, LH92, LH82). The extremely low *ZmMYB30* expressions found in 62R and LH82 suggested the importance of MBS *cis*-elements in regulating gene expression, which was verified by a site-specific mutation in the MBS motif. SNP (-432) was found only in the NC250 line and although it did not impair any putative *cis*-element, the *ZmMYB30* expression in NC250 also was extremely low and similar to the expressions in 62R and LH82. Whether the T→C transition in SNP (-432) was involved in decreasing *ZmMYB30* expression in NC250 needs further verification.

In a previous study, we found that overexpression of *ZmMYB30* in *Arabidopsis* enhanced the salinity tolerance of transgenic *Arabidopsis* seedlings (Chen *et al.* 2018), which suggested *ZmMYB30* may be a key player in the maize stress response. The present results further support our previous conclusion. The *ZmMYB30* expression varied greatly among the inbred lines. For example, between NC250/62R and MBST/H99, the relative expressions varied by hundreds of times. We also determined the expressions of three stress-responsive genes, *ZmSOS1*, *ZmSOS2*, and *ZmABF3*, in different inbred lines. *ZmSOS1* (GRMZM2G098494), *ZmSOS2* (GRMZM2G171507), and *ZmABF3* (GRMZM2G157722) are homologs of *AtSOS1* (*At2g01980*), *AtSOS2* (*At5g35410*), and *AtABF3* (*At4g34000*), respectively. *AtSOS1* encodes a plasma membrane-localized Na⁺/H⁺ antiporter that functions in the extrusion of toxic Na⁺ from cells and is essential for plant salt tolerance (Pehlivan *et al.* 2016). The *AtSOS2* encodes a member of the calcineurin B-like (CBL)-interacting protein kinase family that activates the plasma membrane Na⁺/H⁺ antiporter *SOS1* to bring about sodium ion homeostasis and salt tolerance (Guo *et al.* 2004). The *AtABF3* encodes an ABA-responsive element-binding protein that is similar to the TFs and is expressed in response to stress and ABA (Kerr *et al.* 2018).

The expressions of *ZmSOS1*, *ZmSOS2*, and *ZmABF3* were correlated with the *ZmMYB30* expressions. In the inbred lines 62R and NC250, which had low *ZmMYB30* expression, the expressions of these three genes also were low. The salinity treatment increased the expressions of *ZmMYB30* and the three stress-responsive genes in all the inbred lines tested, except PHR32. In PHR32, the expression of *ZmMYB30* and three stress-responsive genes was not induced by salinity stress, but the expressions of all four genes were higher than their expressions in B73 under normal conditions (Fig. 4A,B).

In future studies, we plan to further investigate the roles of *ZmMYB30* by phenotypic and physiological analyses of stress tolerance in inbred maize lines that have shown enormous differences in *ZmMYB30* expressions, such as those between NC250 and H99. Comparative transcriptome analysis between NC250 and H99 will help to uncover new transcriptional mechanisms of *ZmMYB30*.

References

- Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K.: *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. - *Plant Cell* **15**: 63-78, 2003.
- Baldoni, E., Genga, A., Cominelli, E.: Plant MYB transcription factors: their role in drought response mechanisms. - *Int. J. mol. Sci.* **16**: 15811-15851, 2015.
- Chen, Y.H., Li, H.J., Shi, D.Q., Yuan, L., Liu, J., Sreenivasan, R., Baskar, R., Grossniklaus, U., Yang, W.C.: The central cell plays a critical role in pollen tube guidance in *Arabidopsis*. - *Plant Cell* **19**: 3563-3577, 2007.
- Chen, Y.H., Cao, Y.Y., Wang, L.J., Li, L.M., Yang, J., Zou, M.X.: Identification of MYB transcription factor genes and their expression during abiotic stresses in maize. - *Biol. Plant.* **62**: 222-230, 2018.
- Cheng, M.C., Liao, P.M., Kuo, W.W., Lin T.P.: The *sArabidopsis* ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different *cis*-acting elements in response to different stress signals. - *Plant Physiol.* **162**: 1566-1582, 2013.
- Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X., Agarwal, M., Zhu, J.K.: ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. - *Genes Dev.* **17**: 1043-1054, 2003.
- Dhadi, S.R., Deshpande, A., Ramakrishna, W.: A novel non-wounding transient expression assay for cereals mediated by *Agrobacterium tumefaciens*. - *Plant mol. Biol. Rep.* **30**: 36-45, 2012.
- Garg, B., Lata, C., Prasad, M.: A study of the role of gene *TaMYB2* and an associated SNP in dehydration tolerance in common wheat. - *Mol. Biol. Rep.* **39**: 10865-10871, 2012.
- Guiltinger M.J., Marcotte, W.R., Quatrano, R.S.: A plant leucine zipper protein that recognizes an abscisic acid response element. - *Science* **250**: 267-271, 1990.
- Guo, H., Wang, L., Yang, C., Zhang, Y., Zhang, C., Wang, C.: Identification of novel *cis*-elements bound by BpMYB46 involved in abiotic stress responses and secondary wall deposition. - *J. integr. Plant Biol.* **60**: 1000-1014, 2018.
- Guo, Y., Qiu, Q.S., Quintero, F.J., Pardo, J.M., Ohta, M., Zhang, C., Schumaker, K.S., Zhu, J.K.: Transgenic evaluation of activated mutant alleles of *SOS2* reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in *Arabidopsis thaliana*. - *Plant Cell* **16**: 435-449, 2004.
- Huang, G.T., Ma, S.L., Bai, L.P., Zhang, L., Ma, H., Jia, P., Liu, J., Zhong, M., Guo, Z.F.: Signal transduction during cold, salt, and drought stresses in plants. - *Mol. Biol. Rep.* **39**: 969-987, 2012.
- Jaradat, M.R., J.A. Feurtado, D. Huang, Y. Lu, and A.J. Cutler.: Multiple roles of the transcription factor AtMYB1/AtMYB44 in ABA signaling, stress responses, and leaf senescence. - *BMC Plant Biol.* **13**: 1-19, 2013.
- Kerr, T.C.C., Abdel-Mageed, H., Aleman, L., Lee, J., Payton, P., Cryer, D., Allen, R.D.: Ectopic expression of two AREB/ABF orthologs increases drought tolerance in cotton (*Gossypium hirsutum*). - *Plant Cell Environ.* **41**: 898-907, 2018.
- Kim, J.H., N.H. Nguyen, C.Y. Jeong, N.T. Nguyen, S.-W. Hong, H. Lee.: Loss of the R2R3 MYB, AtMyb73, causes hyper-induction of the *SOS1* and *SOS3* genes in response to high salinity in *Arabidopsis*. - *J. Plant Physiol.* **170**: 1461-1465, 2013.
- Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., Rombauts, S.: PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. - *Nucl. Acids Res.* **30**: 325-327, 2002.
- Liu, S., Wang, X., Wang, H., Xin, H., Yang, X., Yan, J., Li, J., Tran, L.S.P., Shinozaki, K., Yamaguchi-Shinozaki, K., Qin, F.: Genome-wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L. - *PLoS Genetics* **9**: e1003790-e1003790, 2013.
- Mao, H., Wang, H., Liu, S., Li, Z., Yang, X., Yan, J., Li, J., Tran, L.S.P., Qin, F.: A transposable element in a *NAC* gene is associated with drought tolerance in maize seedlings. - *Nature Commun.* **6**: 8326-8326, 2015.
- Pehlivan, N., Sun, L., Jarrett, P., Yang, X., Mishra, N., Chen, L., Kadioglu, A., Shen, G., Zhang, H.: Co-overexpressing a plasma membrane and a vacuolar membrane sodium/proton antiporter significantly improves salt tolerance in transgenic *Arabidopsis* plants. - *Plant Cell Physiol.* **57**: 1069-1084, 2016.
- Persak, H., A. Pitzschke.: Tight interconnection and multi-level control of *Arabidopsis* MYB44 in MAPK cascade signalling. - *PLoS ONE.* **8**: e57547, 2013.
- Roy, S. : Function of MYB domain transcription factors in abiotic stress and epigenetic control of stress response in plant genome. - *Plant Signal. Behav.* **11**: e1117723-e1117723, 2015.
- Samad, A.F.A., Sajad, M., Nazaruddin, N., Fauzi, I.A., Murad, A.M.A., Zainal, Z., Ismail, I.: MicroRNA and transcription factor: key players in plant regulatory network. - *Front. Plant Sci.* **8**: 565-565, 2017.
- Stockinger, E.J., Gilmour, S.J., Thomashow, M.F.: *Arabidopsis thaliana* *CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. - *Proc. nat. Acad. Sci. USA* **94**: 1035-1040, 1997.
- Sun, Y., Dinnyes, J.R.: Q&A??: How do gene regulatory networks control environmental responses in plants? - *BMC Biol.* **16**: 38-38, 2018.
- Tan, W., Zhang, D., Zhou, H., Zheng, T., Yin, Y., Lin, H.: Transcription factor HAT1 is a substrate of SnRK2.3 kinase and negatively regulates ABA synthesis and signaling in *Arabidopsis* responding to drought. - *PLoS Genet.* **14**: e1007336-e1007336, 2018.
- Tran, L.S.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K.: Isolation and functional analysis

- of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter. - *Plant Cell* **16**: 2481-2498, 2004.
- Urban, A., Neukirchen, S., Jaeger, K.E.: A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR -. *Nucl. Acids Res.* **25**: 2227-2228, 1997.
- Viana, V.E., Busanello, C., Da Maia, L.C., Pegoraro, C., Costa de Oliveira, A.: Activation of rice WRKY transcription factors: an army of stress fighting soldiers? - *Curr. Opin. Plant Biol.* **45**: 268-275, 2018.
- Wang, X., Wang, H., Liu, S., Ferjani, A., Li, J., Yan, J., Yang, X., Qin, F.: Genetic variation in ZmVPP1 contributes to drought tolerance in maize seedlings. - *Nat.Genet.* **48**: 1233-1241, 2016.
- Xiang, Y., Sun, X., Gao, S., Qin, F., Dai, M.: Deletion of an endoplasmic reticulum stress response element in a *ZmPP2C-A* gene facilitates drought tolerance of maize seedlings. - *Mol. Plants* **10**: 456-469, 2017.
- Xu, R., Wang, Y., Zheng, H., Lu, W., Wu, C., Huang, J., Yan, K., Yang, G., Zheng, C.: Salt-induced transcription factor MYB74 is regulated by the RNA-directed DNA methylation pathway in *Arabidopsis*. - *J. exp.Bot.* **66**: 5997-6008, 2015.
- Yamaguchi-Shinozaki, K., Shinozaki, K.: Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. - *Annu. Rev. Plant Physiol.* **57**: 781-803, 2006.
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W.: A greedy algorithm for aligning DNA sequences. - *J. Comput. Biol.* **7**: 203-214, 2000.
- Zhao, Y., Cheng, X., Liu, X., Wu, H., Bi, H., Xu, H.: The wheat MYB transcription factor TaMYB31 is involved in drought stress responses in *Arabidopsis*. - *Front. Plant Sci.* **9**: 1426, 2018.
- Zhou, L.J., Zhang, C.L., Zhang, R.F., Wang, G.L., Li, Y.Y., Hao, Y.J.: The SUMO E3 ligase MdSIZ1 targets MdbHLH104 to regulate plasma membrane H⁺-ATPase activity and iron homeostasis. - *Plant Physiol.* **179**: 88-106, 2019.
- Zhu, J.K.: Abiotic stress signaling and responses in plants. - *Cell* **167**: 313-324, 2016.