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

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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...
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 (ARE), and CRT/DRE, MYC, and MYBRE cis-elements, and the corresponding TFs that bind to them also have been identified (Guilminan 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 cordon 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 μmol m−2 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 μmol m−2 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 (Thermo Scientific, 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 pJE1.2/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, three 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 3S 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 pB1101.1 vector through the HindIII and BamHI 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 cm² 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 μM acetosyringone (20 mm², Sigma-Aldrich, St. Louis, USA) and Silwet L-77 (10 mm², 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 grown in a sterile 15-mm Petri dish and incubated for 12 h with 15 - 20 cm 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 T-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 μg total RNA from the different seedlings was used for reverse transcription with a PrimeScript™ 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 2x SYBR Green PCR Master Mix (Roche, Basel, Switzerland), 2.0 mm² of cDNA (10-fold dilution), and 1 mm² of gene-specific primers (2.0 μM) 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^(-ΔΔCt) 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 1461-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
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 (-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

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 cis-acting element in promoter and enhancer regions |
| cis element | CGTCA | -1275 | cis-acting regulatory element involved in MeJA-responsiveness |
| TC-rich repeats | ATTCTCTAAC | -981(-) | cis-acting element involved in defense and stress responsiveness |
| I-box | GATAAGGTG | -1119 | part of a light responsive element |
| ARE | AAACCA | -1264, -1107, -926, -1001(-) | cis-acting regulatory element essential for the anaerobic induction |
| ABRE | ACGTG | -677, -511(-) | cis-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 | AAGCAG | -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                |          |             |                    |
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). In the GUS staining experiments, apart from the positive control 35S promoter, which all parts of 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 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. 2.** The sequence of DNA and putative cis-acting elements in ZmMYB30 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 HindIII and BamHI 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 (HindIII and BamHI). 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 Arabidopsis seedlings without treatment were 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 (D5 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 verifications.

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 (Pehlivian 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.

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