IDENTIFICATION OF TRANSCRIPTION FACTOR ZMZAT8 INVOLVED IN ABSCISIC ACID REGULATION PATHWAY OF STARCH SYNTHESIS IN MAIZE ENDOSPERM

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Abstract

Maize starch, which is closely related with the yield and the quality of the produced maize, has wide applications. Even though the main pathway of starch biosynthesis has been identified, the full array of genes involved and the mechanisms regulating their expression are still unclear. In this study, a new gene, named ZmZAT8, was chosen and cloned as a candidate gene based on our previously published transcriptome data of maize endosperm treated with ABA. Bioinformatics analysis indicated that ZmZAT8 encodes 21.6kD protein comprising 184 amino acids and two QALGGH domains, which is a conserved feature of plant C2H2-type Zinc-finger proteins. ABA could strongly stimulate the expression of ZmZAT8, as confirmed by real-time RT-PCR analysis. Moreover, a series of evaluations regarding the ZmZAT8 were performed, including analyses of subcellular localization, transcription activation, protein-DNA interactions, and transient expression in maize endosperm. The results showed that the ZmZAT8 protein was capable of regulating the expression of Sh2, Bt1, and Wx, which were all key genes involved in starch synthesis. In conclusion, our data suggests that ZmZAT8, a novel ABA-induced C2H2-type zinc-finger transcription factor, may play a positive role in regulating starch synthesis in maize endosperm.

Key words: Maize starch synthesis, ABA, ZmZAT8, Transcription factor.

Introduction

Maize (Zea mays) is one of the most widely distributed food crops in the world. The large geographical extent of its cultivation, its low price, good nutritional value, numerous industrial applications, and the existence of various maize types, make it a widely used grain in the food industry, for livestock feed, and as an industrial raw material. Therefore, maize plays a very important role in global economy (Leff et al., 2004). Starch is the main energy storage material of maize seeds, and is closely associated to the yield and quality of maize. The main synthesis pathway of starch is already well understood; however, the entire array of involved genes and their regulatory mechanisms are still unclear.

Starch biosynthesis in higher plants is orchestrated by the coordinated action of four enzymes: ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch-branching enzyme (SBE), and starch debranching enzyme (DBE) (Hannah, 2005; Hennen-Bierwagen et al., 2009; Smith et al., 1997). AGPase is the first key enzyme, which catalyzes the creation of ADP-glucose from glucose-1-phosphate and ATP. It is composed of two large subunits and two small subunits, encoded by shrunken-2 (Sh2) and brittle-2 (Bt2), respectively (Doan et al., 1999). The transfer of ADP-Glucose from cytosol into seed amyloplasts is facilitated by an adenylate translocator protein, Brittle-1, which is encoded by Bt1 and binds ADP-Glucose through its characteristic KTGGL motif (Shannon et al., 1998). In maize, mutants of the genes encoding the subunits of AGPase, i.e. Sh2 and Bt2, and mutants of the transporter gene Bt1, result in a substantial reduction, about 90%, in starch synthesis (Hannah et al., 2001; Patron et al., 2004).

ADP-Glucose is added to the nonreducing end of an existing glucan chain through the creation of a new alpha 1,4-bond, catalyzed by SS in the case of amylpectin and GBSS in the case of amylose. The waxy gene is a nonfunctional mutant allele of GBSS-I (Fan et al., 2008; Klösgen et al., 1986; Shure et al., 1983). Waxy maize, a type of maize where this mutation was first discovered, had little or no GBSS-I protein and amylose in endosperms. Due to its many peculiar traits, growing waxy maize on a commercial scale requires extra measures compared to standard dent maize (Jobling, 2004; Wang et al., 1995).

Abscisic acid (ABA) is an indispensable phytohormone that plays a regulatory role in plant growth and developmental processes such as seed development, dormancy, and germination; it is involved in stomatal movement and also regulates plant responses to a variety of types of environmental stress (Cao et al., 2011; Cutler et al., 2010; Leung & Giraudat, 1998; Zeevaart & Creelman, 1988). In rice, ABA content has been shown to be significantly correlated with grain filling rate, which is attributed to the ability of ABA to regulate the expression of starch synthesis genes (Mukherjee et al., 2015; Wang et al., 2015; Zhu et al., 2011). In wheat, Mukherjee et al., (2015) reported that ABA could play a role in the regulation of genes related to sucrose transport and its conversion to starch during grain filling. In maize, previous studies conducted in our laboratory demonstrated that ABA could increase the expression of some starch synthesis genes (Chen et al., 2011) and enhance ZmSSI (Zea mays starch synthase I) expression through the binding of the ABI4 transcription factor to a CACCG motif in the ZmSSI promoter (Hu et al., 2012). Generally, many transcription factors have been found to be involved in ABA signaling (Wind et al., 2013), but only a few are reported as being involved in the ABA regulation of the expression of starch synthesis genes.
In this study, a new gene, named ZmZAT8, was identified and cloned using our previously published transcriptome data from maize endosperm treated with ABA. A series of evaluations of ZmZAT8 were performed, including bioinformatic analysis, subcellular localization, transcription activation, protein-DNA interaction, and transient expression in maize endosperm. Our results demonstrate that ZmZAT8 is an ABA-induced gene encoding a novel C2H2-type zinc-protein transcription factor, capable of increasing the expression of Sh2, Btl, and Wx.

Materials and Methods

Plant materials, growth conditions, and transcriptome data: The maize inbred line B73 was grown in the field and was strictly self-pollinated. Ten days after pollination (DAP), developing endosperms was treated with 100 μM ABA for 24 h, RNA isolation, and transient expression. The transcriptome data of the controls and the ABA-treated 10-DAP endosperms were deposited in the NCBI Sequence Read Archive under the accession number SRP068962, and published separately (Huang et al., 2016).

Gene cloning and bioinformatics analysis of ZmZAT8: Many genes with significant differences in expression levels were selected from the control vs. ABA library. For details, refer to the methods section in our previous paper (Huang et al., 2016). One of them, gene sequence GRMZM2G112799, was named ZmZAT8 and was chosen for further analysis (Huang et al., 2016). The total RNA of 10-DAP endosperms treated with ABA was isolated using TRIzol® reagent (Thermo Fisher Scientific, Pittsburgh, PA, USA), and treated with gDNA Eraser (Takara Bio Inc., Otsu, Japan) to remove genomic DNA contamination. Total RNA (1 μg) was used to produce cDNA using the PrimeScript™ RT reagent kit (Takara Bio Inc.). ZmZAT8 was cloned using the KOD enzyme (TOYOBO, Japan) and the following primers: ZmZAT8-F: 5′-ATGTCATGAGCCTCCAGAGAC-3’ and reverse, 5′-CTATACGAGGTTGAGCA GCTG-3’ (the underlined sections of the forward and the reverse primers are BamHI and XbaI sites, respectively). The PCR product was inserted into pMD19-T and digested with BamHI and XbaI. The resultant fragment was ligated into pCAMBIA2300-35S-eGFP, which contained a eGFP (enhanced green fluorescence protein) driven by the CaMV 35S promotor. The resulting construct, pCAMBIA2300-35S-eGFP-ZmZAT8 (35S:: eGFP-ZmZ8), was transiently expressed in onion epidermis cells after biolistic bombardment. Cells bombarded with the pCAMBIA2300-35S-eGFP were used as a control (35S::eGFP). The preparations of plasmid DNA and of the gold particle solution were done according to Hu et al. (2011). After incubation in the dark for 24 h at 28°C, the subcellular localization of the fusion protein was observed using a model NumberA1R/A1 confocal laser scanning microscopy (Nikon).

Expression level of ZmZAT8: To confirm that ABA increases the expression of ZmZAT8, the gene’s cDNA levels in 10-DAP endosperms treated with ABA were measured by real-time PCR, using the following primers: forward, 5′-GCAAGCGGTTCCCGTGTCC-3’ and reverse, 5′-GGTGCATCGTCGTCC-3’. The maize 18S rRNA gene was used as an internal control, using the following primers: forward, 5′-CCTGGGG CTAATTGACTC-3’ and reverse, 5′-GTAGCAGG CTGAGTCC-3’. We used the SYBR® Prime Script™ RT-PCR Kit Perfect Real Time (Takara Bio Inc.), according to the manufacturer’s instructions.

Homologues of ZmZAT8 and phylogenetic analysis in plant species: The homologues sharing high sequence similarities with ZmZAT8 were obtained based on BLAST search in NCBI (http://www.ncbi.nlm.nih.org) using ZmZAT8 sequence as a query. The incorrect or redundant entries were eliminated by comparing the sequences within each species. MEGA 6.0 was used for phylogenetic analysis with other zinc finger proteins. MEGA 6.0 was used for phylogenetic analysis with the neighbor-joining method at default settings based on comparison of 1,000 bootstrap replications.

Subcellular localization

The full-length coding region of ZmZAT8 was amplified by PCR using the following Primers: forward, 5′-GAATTCTAGTTCATACGAGGTTACGAGAC-3’ and reverse, 5′-TCTAGATACGAGCAGGTAGCA GCTG-3’ (the underlined sections of the forward and the reverse primers are BamHI and XbaI sites, respectively). The PCR product was inserted into pMD19-T and digested with BamHI and XbaI. The resultant fragment was ligated into pCAMBIA2300-35S-eGFP, which contained a eGFP (enhanced green fluorescence protein) driven by the CaMV 35S promotor. The resulting construct, pCAMBIA2300-35S-eGFP-ZmZAT8 (35S:: eGFP-ZmZ8), was transiently expressed in onion epidermis cells after biolistic bombardment. Cells bombarded with the pCAMBIA2300-35S-eGFP were used as a control (35S::eGFP). The preparations of plasmid DNA and of the gold particle solution were done according to Hu et al. (2011). After incubation in the dark for 24 h at 28°C, the subcellular localization of the fusion protein was observed using a model NumberA1R/A1 confocal laser scanning microscopy (Nikon).

Table 1. The information of Bioinformatic analysis software for ZmZAT8.

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<tr>
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Transactivation activity assay: The full-length cDNA was amplified by PCR using the following primers: forward, 5’-GGATCCATGGTCAAGCGTTACGAAC-3’ and reverse, 5’-GGATCCATGGTCAAGCGTTACGAAC-3’ (the underlined sections in the forward and the reverse primer sequences are BamHI and EcoRI restriction sites, respectively). The PCR product was inserted into the pMD19-T cloning vector. After digestion with BamHI and EcoRI, the resulting fragment was ligated into the pGBKTK7 DNA-binding domain vector (Takara Bio Inc.) and named pGBKTK7-ZmZAT8 after identification.

The transactivation activity assay was performed using the GAL4 one-hybrid system. The constructs were transformed into yeast strain AH109, with pGBKTK7-GAL4 AD as positive control, and pGBKTK7 and AH109 as negative controls. The transformants were screened on SD−Trp medium, and positive clones were identified using PCR. The transformed yeast colonies were grown to an OD600 of 0.5, then diluted and drop-plated on synthetic defined (SD) minimal media lacking tryptophan and histidine (SD−Trp−His), with 20 µL from a 20 mg mL−1 X-α-gal solution. Yeast cells were cultivated at 28°C for 3 days to test transcription activation (Hossain et al., 2010).

Transient expression assays in maize endosperm: The full-length coding region of ZmZAT8 was amplified by PCR using the following Primers: forward primer, 5’-GGATCCATGGTCAAGCGTTACGAAC-3’ and reverse primer, 5’-ACCGGTCTATACGACGAGGTT GACGACTG-3’ (the underlined sections of the forward and reverse primer sequences are BamHI and MluI restriction sites, respectively). After treatment with BamHI and MluI, the fragments were ligated into the MCS of the pBI221 vector, where they were driven by the ubiquitin promoter. After identification, the loaded vector was named Ubi-ZmZAT8. We also made use of Sh2-LUC, Wx-LUC and Bt1-LUC provided by our lab, containing the first intron of the Adh1 gene in order to enhance promoter activity without altering promoter specificity, in these constructs, the promoters of Sh2, Wx and Bt1 drove LUC reporter gene transcription, respectively.

Ten DAP maize kernels were surface-sterilized with 75% (v/v) ethanol. Then the endosperm were isolated and cultivated on MS medium (Murashige and Skoog basic salt mixture) containing 1% agar and 10% sucrose for 4 h prior to bombardment. A helium biolistic gun transformation system (Bio-Rad, Hercules, CA, USA) was used to deliver gold particles coated with the desired constructs. Each independent experiment consisted of four replicates. The bombardered endosperms were then cultivated for 24 h in order to analyze the LUC reporter gene expression. The plasmid pBI221 vector, containing the GUS gene driven by a maize ubiquitin promoter, was used as a control in order to correct for transfection efficiency. The experiment was performed according to a protocol developed in our laboratory (Hu et al., 2011).

Yeast one-hybrid assay: The yeast-one hybrid assay is one of the most common methods for in vitro analysis of protein-DNA binding. To verify the ability of the ZmZAT8 protein to bind on the promoters of Sh2, Wx, and Bt1 in vitro, the coding region of ZmZAT8 was ligated into the pGADT7-Rec2 vector (Takara Bio Inc.), named pGADT7-Rec2-ZmZAT8 after identification. The promoters of Sh2, Wx, and Bt1 were cloned and inserted into pHis2 plasmids (Takara Bio Inc.), and respectively named pHis2-Sh2, pHis2-Wx, and pHis2-Bt1, which had been constructed in our lab before (supplement 1). The desired combinations of recombinant expression vectors were co-transformed into the Y187 yeast strain. The transformed yeast cells were first screened on the SD−Trp−Leu medium, and the positive clones were transferred on the detect type medium, SD−Trp−Leu−His+/100 mM 3AT (3-amino-1, 2, 4-triazole). The plasmids pHis2-Sh2/pGADT7-Rec2, pHis2-Wx/ pGADT7-Rec2, and pHis2-Bt1/ pGADT7-Rec2 were respectively used as the negative controls. Yeast cells were cultivated at 28°C for 3 days to test the growth status of yeast (Hossain et al., 2010).

Results

Gene cloning and bioinformatics analysis of ZmZAT8: The full-length cDNA of ZmZAT8 was amplified by RT-PCR (supplement 2), and the PCR product was attached to the pMD19-T vector. Sequencing revealed that the cDNA sequence of ZmZAT8 was consistent with the GenBank report (supplement 3). Using the Expasy Proteomics Tools, we determined that ZmZAT8 contained a 555 bp open reading frame (ORF), which encoded a protein of 184 amino acids, with an isoelectric point of 5.09 and a putative molecular weight of 21.86 kD (Fig. 1). The ZmZAT8 protein mainly consisted of the α-helix finger structure (Fig. 2), and was made up of a three-stranded β-sheet and three α-helices (supplement 4). The ZmZAT8 protein has two typical structural domains of C2H2 zinc-finger proteins, as predicted by ExPaSy Protomics Tools. The full-length cDNA of ZmZAT8 was registered in GenBank (Accession No.: KF146208).

CARE program, and the results showed that the ZmZAT8 protein belongs to the C2H2 zinc-finger family. Sequence alignment using ClustalW software showed that ZmZAT8 contained a hydrophilic amino acid group L-box and a DLN-box (Fig. 3). Leu residues are enriched in the L-box and the DLN-box, and may play an important role in the protein-recognition interactions and maintenance of protein folding structure (Sakamoto et al., 2000). Together, the above results strongly suggest that ZmZAT8 protein belongs to the C2H2 ZFPs transcription factor family.

The ZmZAT8 promoter (2000 bp) was analyzed using the Plant-CARE program, and the results showed that the promoter contained several cis-acting elements, specifically five ABREs, one RY-element, and one SKn-1 motif (Table 2). ABREs (ABA responsive elements) are the main cis-acting elements mediating ABA-induced transcription, the RY-element is involved in seed-specific regulation, and the SKn-1 motif is involved in endosperm expression. Therefore, the ZmZAT8 gene may be involved in the regulation of endosperm growth by the ABA pathway.

Phylogenetic (NJ) tree was constructed based on the amino acid sequences of ZmZAT8 and other ZFPs transcription factors (supplement 6) using MEGA6.0 software (Fig. 4). In maize, ZmZAT8, ZmZnF1 (GRMZM5G836222) and ZmZnF2 (GRMZM2G116079) clustered in a same branch, and ZmZnF2 had higher sequence similarity than ZmZnF1 with ZmZAT8. Moreover, ZmZAT8 may take part in similar regulatory mechanisms as OS03G0820300 and OS01G0839100.
Fig. 1. Deduced primary protein structure of ZmZAT8
Sequence of ZmZAT8 full length cDNA nucleotides and amino acids, square frames are two conserved motifs of ZmZAT8, and highlight words are C2H2 zinc finger protein special conserved amino acids motif QALGGH, L-box is underlined in black, DLN-box is underlined in red.

Table 2. Putative function of the cis-acting element in the ZmZFP1 promoter predicted by Plant-CARE.

<table>
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<tr>
<th>The name of cis-acting element</th>
<th>Number</th>
<th>Site (bp)</th>
<th>Sequence</th>
<th>Function</th>
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<td>ABRE</td>
<td>5</td>
<td>175 (+) 684 (+) 1076 (+) 1254 (+) 1684 (+)</td>
<td>ACGTG</td>
<td>Abscisic acid-response</td>
</tr>
<tr>
<td>RY-element</td>
<td>1</td>
<td>965 (+)</td>
<td>CATGCATG</td>
<td>Seed-specific regulation</td>
</tr>
<tr>
<td>SKn-1 motif</td>
<td>2</td>
<td>1010 (+), 1516 (+)</td>
<td>GTCAT</td>
<td>Endosperm expression</td>
</tr>
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</table>

Expression of ZmZAT8 in endosperm after ABA treatment: According to transcriptome data, the expression of ZmZAT8 in endosperms treated with ABA treatment was increased by 2.82 times compared to controls. Real-time PCR demonstrated that the expression of ZmZAT8 in the AB-treated endosperms was 2.36 times higher than that in the controls (Fig. 5), which was consistent with the transcriptome data. We concluded that exogenous ABA could significantly up-regulate the expression of ZmZAT8.

Subcellular localization: The pCAMBIA2300-35S-eGFP-ZmZAT8 construct and the pCAMBIA2300-35S-eGFP control were introduced into onion epidermal cells via particle bombardment. As shown in Figure 6, fluorescence detection by confocal microscopy revealed that the 35S::eGFP-ZmZAT8 fusion protein was localized in the nucleus, whereas the control 35S::eGFP was localized in both cytoplasm and the nucleus. This suggests that ZmZAT8 might be a transcription factor.

Transcription activation: The construct pGBK7-ZmZAT8 was transformed into yeast strain AH109 cells using the LiAc method. The cells were screened on SD minimal medium (SD/-Trp/-His) with 20 µL (20 mg/mL) X-α-gal. Positive clones were identified using PCR and were further cultivated on SD/-Trp/-His/+10 mmol 3-AT/+X-α-gal mediums. We found that the yeast cells harboring pGBK7-ZmZAT8 were able to grow and change the color of the medium to blue, indicating that ZmZAT8 possessed transcription activator activity (Fig. 7).
Transient expression assays in maize endosperm: The construct Ubi-ZmZAT8 was co-transformed with Sh2-LUC, Wx-LUC or Bt1-LUC into 10-DAP maize endosperms in order to analyze the interactions of ZmZAT8 with the promoters of Sh2, Wx, and Bt1, respectively (Fig. 8A). The results showed that the LUC/GUS ratio was about 1.98 when Ubi-ZmZAT8 was co-transformed with either Wx-LUC or Bt1-LUC (Fig. 8B), with a LUC/GUS ratio of 1 in controls. Interestingly, the ratio of LUC/GUS in endosperms co-transformed with Ubi-ZmZAT8 and Sh2-LUC was even higher, up to 3.28, with a 0.5 ratio in controls (Fig. 8B). We concluded that the ZmZAT8 protein was able to increase the expression of Wx, Bt1 and, especially, Sh2 gene.

Protein-DNA interaction: Yeast one-hybrid experiments were performed to determine the ability of ZmZAT8 to bind to the promoters of Sh2, Wx, and Bt1 (Table 1). The respective promoters were co-transformed into yeast strain Y187, and yeast cells were screened using a synthetic dropout nutrient medium. As shown in Figure 9, yeasts harboring the constructs pHis2-Sh2/pGADT7-Rec2-ZmZAT8, pHis2-Wx/pGADT7-Rec2-ZmZAT8 or pHis2-Bt1/pGADT7-Rec2-ZmZAT8, could grow on SD−Trp−/His− medium. The positive clones harboring the different constructs were further screened on SD−Trp−/Leu−/His−/100 mM 3AT medium. The cells containing pHis2-Sh2/pGADT7-Rec2-ZmZAT8 or pHis2-Wx/pGADT7-Rec2-ZmZAT8 continued to grow, which strongly suggested that the ZmZAT8 protein could directly bind to the promoters of sh2 and Wx. In contrast, the cells harboring pHis2-Bt1/pGADT7-Rec2-ZmZAT8 were unable to grow on the selection medium, revealing that ZmZAT8 protein could not interact directly with the promoter of Bt1.

Fig. 3. Multiple alignments of ZmZAT8 proteins
Alignment of ZmZAT8 with finger protein of other plants. Multiple alignment of these zinc finger proteins was made with the Clustal W program, red rectangular boxes are characteristic amino acid sequences (two zinc fingers, DLN-box, L-box); positions containing identical residues are shaded in red and blue, whereas conservative residues are in black. These sequences were retrieved from NCBI. The species name, species type, and GenBank accession numbers, ZmZAT8 (Zea mays XP_008657926), XP_008674848 (Zea mays XP_008674848), AAL76091 (Oryza sativa Japonica Group AAL76091), XP_003567258 (Brachypodium distachyon XP_003567258) and XP_015638878 (Oryza sativa Japonica Group XP_015638878). – Discuss.
Fig. 4. Phylogenetic tree analysis of ZmZAT8
Phylogenetic tree analysis of ZmZAT8 (GRMZM2G112799) based on amino acids sequences and built using MEGA6.0 software. The tree was generated using the neighbor-joining method with bootstrap support by 1000 replicates. The GenBank accession numbers and amino acids sequences are listed in supplement 6.

Fig. 5. Real-time qPCR analysis of ZmZAT8
Expression of ZmZAT8 was upregulated by ABA treatment. Ten DAP endosperms were treated with 100 µM ABA for 24 h.

Fig. 6. Subcellular localization analysis of ZmZAT8
Subcellular localization analysis of ZmZAT8 in onion epidermal cells. Fluorescence microscopy of onion epidermal cells expressing either eGFP, eGFP-ZmZAT8 as indicated (scale bar=100 um).

Fig. 7. Transactivation activity assay results for ZmZAT8
The structure of the pGBK7-ZmZAT8 plasmid: The growth of transformed yeast cells on SD-Trp, SD-Trp-His-Ura and SD-Trp-His-Ura+10mg/mL X-a-gal medium, respectively. From the left to the right, the name of map is the corresponding pGBK7-GAL4 AD was the positive control, pGBK7, pGBK7-Lam, and pGBK7-53 were negative controls.
A regulation of genes expression in plant development and responses to environmental stress. Recent research has shown that many transcription factors involved in mediating the effects of abscisic acid (ABA) signaling in Arabidopsis are significantly increased by ABA (Yu, et al., 2015). Therefore, it is speculated that ABA in maize kernels are significantly increased by ABA (Yu, et al., 2009). 

**Zinc finger proteins play various important roles in plant development and responses to environmental stress. ZmZnF1 is induced by ABA, dehydration and high concentration of NaCl in maize kernels.** Transient assay results suggest that ZmZnF2 enhances ABA-responsive gene expression in the presence of viviparous 1 (VIP) and may be involved in the ABA signaling pathway (Huang, et al., 2016). In this study, a novel ABA-induced gene, ZmZAT8, was identified in maize endosperm and cloned. Bioinformatic analysis revealed that ZmZAT8 encodes a protein that belongs to the group of C2H2-type zinc-finger proteins, and may play a regulatory role in endosperm development. The phylogenetic (NJ) tree results indicated that transcription factors possessed similar functions with separate branch. OS03G0820300 and OS01G0839100 are induced by ABA, ROS and stress signaling (Huang, et al., 2007). Moreover, the expression of ZmZnF1 and ZmZnF2 transcription factors from maize kernels are significantly increased by ABA (Yu, et al., 2015). Recent research demonstrated that AYY1 is another zinc-finger protein and can directly upregulate the expression of the AB4 repressor 1 (ABR1) gene. Furthermore, its expression is regulated by ABI4, whereas ABI4’s effect can be antagonized by ABR1 (Li, et al., 2016). Zinc finger protein 3 (ZFP3) can negatively regulates the expression of ABI4, disturbing the ABA signaling in Arabidopsis seed sprout and plant growth (Joseph, et al., 2014). Our previous research reported that the ABI4 transcription factor could enhance the expression of SSI, and improve starch synthesis in maize. Although many transcription factors involved in mediating the effects of the ABA signaling pathway on seed development have already been identified, only a few were described as being involved in the ABA regulation of genes expression in composing starch.

**Discussion**

It is widely believed that RNA-seq is a new technology for accurately analyzing transcription levels, detecting sequence variation and identifying novel transcript sequences, abilities that may allow it to replace microarrays (Hansey, et al., 2012; Li & Dewey, 2011). Here, the induction of ZmZAT8 expression in endosperm after ABA treatment was confirmed using both real-time PCR and transcriptome data. This consistence indicates that transcriptome data are indeed accurate and reliable.

**Fig. 8.** Transient assay for the interaction between ZmZAT8 and the promoter of Sh2, Wx and Bt1 in maize endosperm. ZmZAT8 enhances promoter activity of Bt1, Sh2, Wx, (A) Diagram of the reporter construct, internal control construct and reporter construct used in the experiment. (B) Ubi-ZmZAT8 relative activity was measured using Sh2-LUC, Wx-LUC, Bt1-LUC, as respective controls. Luc was the luciferase gene, and Gus was the β-glucuronidase gene. The data are given as the mean ± SE of four replicates. The asterisk symbol (*) indicates a significant difference between the Ubi-ZmZAT8 and control treatments (p<0.05).

**Fig. 9.** Identification of Sh2, Wx and Bt1 regulated by ZmZAT8 with yeast one hybrid assay. (A) Schematic structure of yeast expression construct pGADT7-Rec2-ZmZAT8 and reporter construct pHis2-Promoter Sh2, Wx and Bt1 promoter; (B) Growth results on defect type medium SD/-Trp/-Leu, SD/-Trp/-Leu/-His medium containing 50 mM 3-AT and SD/-Trp/-Leu/-His medium containing 100 mM 3-AT, which in a series of 10-fold dilutions. pHis2-Sh2/pGADT7-Rec2, pHis2-Wx/pGADT7-Rec2 and pHis2-Bt1/pGADT7-Rec2 were used as negative controls.

**actuate Bt1 expression. This suggests that ZmZAT8 may function together with other synergistic regulators, as has been shown in other systems (Saito, et al., 2016).**

Studies have shown that many transcription factors are involved in the ABA signaling pathway and play a crucial role in it. The expression of ZmZnF1 and ZmZnF2 transcription factors from maize kernels are significantly increased by ABA (Yu, et al., 2015). Recent research demonstrated that AYY1 is another zinc-finger protein and can directly upregulate the expression of the AB4 repressor 1 (ABR1) gene. Furthermore, its expression is regulated by ABI4, whereas ABI4’s effect can be antagonized by ABR1 (Li, et al., 2016). Zinc finger protein 3 (ZFP3) can negatively regulates the expression of ABI4, disturbing the ABA signaling in Arabidopsis seed sprout and plant growth (Joseph, et al., 2014). Our previous research reported that the ABI4 transcription factor could enhance the expression of SSI, and improve starch synthesis in maize. Although many transcription factors involved in mediating the effects of the ABA signaling pathway on seed development have already been identified, only a few were described as being involved in the ABA regulation of genes expression in composing starch.

In this study, the ZmZAT8 protein is identified as a novel transcription factor involved in ABA signal pathway and starch synthesis. Moreover, ZmZAT8 protein may simultaneously regulate multiple gene expressions involved in starch synthesis, such as Sh2, Wx, and Bt1 gene. The introduction of transgenes into maize is difficult due to a low transformation rate and long breeding time. Nevertheless, the production of stable transgenic lines is the most important technique for testing target gene function. Therefore, our further studies will focus on the production of stable transgenic lines of ZmZAT8 gene to investigate its effect on starch content, whether it has potential prospects in maize breeding development.

Compliance with Ethical Standards
Acknowledgments

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References