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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 *Bt*2, 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 amylopectin 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 *ZmSS1* (*Zea mays* starch synthase I) expression through the binding of the ABI4 transcription factor to a CACCG motif in the *ZmSS1* 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*, *Bt1*, 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′-ATGGTCATGACGCTTACGAGACAC-3′; *ZmZAT8*-R: 5′-CTATACGAGCAGGTTGAGCAGCTG-3′. The primers were designed using the Primer 5 software (PRIMER-E Ltd., Ivybridge, UK), according to NCBI gene sequences, and synthesized by Thermo Fisher Scientific. The PCR amplification product was ligated into the pMD19-T vector (Takara Bio Inc.) and sequenced by Majorbio Co. (China). After the identification of *ZmZAT8*, its bioinformatics analysis was performed using various software, which was presented in Table 1.

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′-GAATTCATGGTCATGACGCTTACGAGACAC-3′ and reverse, 5′-TCTAGATACGAGCAGGTTGAGCA GCTG-3′ (the underlined sections of the forward and the reverse primers are *BamH*I and *Xba*I sites, respectively). The PCR product was inserted into pMD19-T and digested with *BamH*I and *Xba*I. The resultant fragment was ligated into pCAMBIA2300-35S-eGFP, which contained a eGFP (enhanced green fluorescence protein) driven by the CaMV 35S promoter. The resulting construct, pCAMBIA2300-35S-eGFP-*ZmZAT8* (35S:: eGFP-*ZmZAT8*), 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′-GCAAGCGGTTCCCGTCGTTC-3′ and reverse, 5′-GGCTGCCATCGTCGTCGTCC-3′. The maize *18S* rRNA gene was used as an internal control, using the following primers: forward, 5′-CCTGGG CTAATTGACTC-3′ and reverse, 5′-GTTAGCAGG CTGAGTCCG-3′. We used the SYBR® Prime Script™ RT-PCR Kit Perfect Real Time (Takara Bio Inc.), according to the manufacturer's instructions.

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

No	Item	Software name	Predicted website
1.	Gene sequence	ExPASy proteomics	http://www.expasy.ch/tools/
2.	Secondary structure	Psipred	http://bioinf.cs.ucl.ac.uk/psipred/
3.	Tertiary structure	SWISS-MODEL	http://swissmodel.expasy.org/
4.	Search homology protein	NCBI	http://www.ncbi.nlm.nih.gov/BLAST/
5.	Protein domain	Expasy-Prosite and SMART	http://prosite.expasy.org/ and http: //smart.embl-heidelberg.de/
6.	Prediction of the cis-acting element	Plant-CARE	http://bioinformatics.psb.ugent.be/webtools/plantcare/htmL/

Transactivation activity assay: The full-length cDNA was amplified by PCR using the following primers: forward, 5′-GAATTCATGGTCATGACGCTTACGAG ACAC-3′ and reverse, 5′-GGATCCCTATACGAGCAGG TTGAGCAGCTG-3′ (the underlined sections in the forward and the reverse primer sequences are *BamH*I and *Eco*RI restriction sites, respectively). The PCR product was inserted into the pMD19-T cloning vector. After digestion with *BamH*I and *Eco*RI, the resulting fragment was ligated into the pGBKT7 DNA-binding domain vector (Takara Bio Inc.) and named pGBDKT7- *ZmZAT8* after identification.

The transactivation activity assay was performed using the GAL4 one-hybrid system. The constructs were transformed into the yeast strain AH109, with pGBKT7-GAL4 AD as positive control, and pGBKT7 and AH109 as negative controls. The transformants were screened on SD/−Trp medium, and positive clones were confirmed using PCR. The transformed yeast colonies were grown to an OD_{600} 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′-GGATCCATGGTCATGACGCTTACGAGACAC-3′ and reverse primer, 5′-ACGCGTCTATACGAGCAGGTT GAGCAGCTG-3′ (the underlined sections of the forward and reverse primer sequences are *BamH*I and *Mlu*I restriction sites, respectively). After treatment with *BamH*I and *Mlu*I, 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 AdhI 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 endosperms 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 bombarded 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 long 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 21.6kD (Fig. 1). The *ZmZAT8* protein mainly consisted of the α-helix structure (Fig. 2), and was made up of a three-stranded β-sheet and three α-helixes (supplement 4). The ZmZAT8 protein has two typical structural domains of C2H2 zinc-finger proteins, as predicted by ExPASy-Prosite and SMART software (supplement 5). Each domain contains a QALGGH motif, which is the typical characteristic of the plant zinc-finger protein 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 (2,000 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.

A 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.

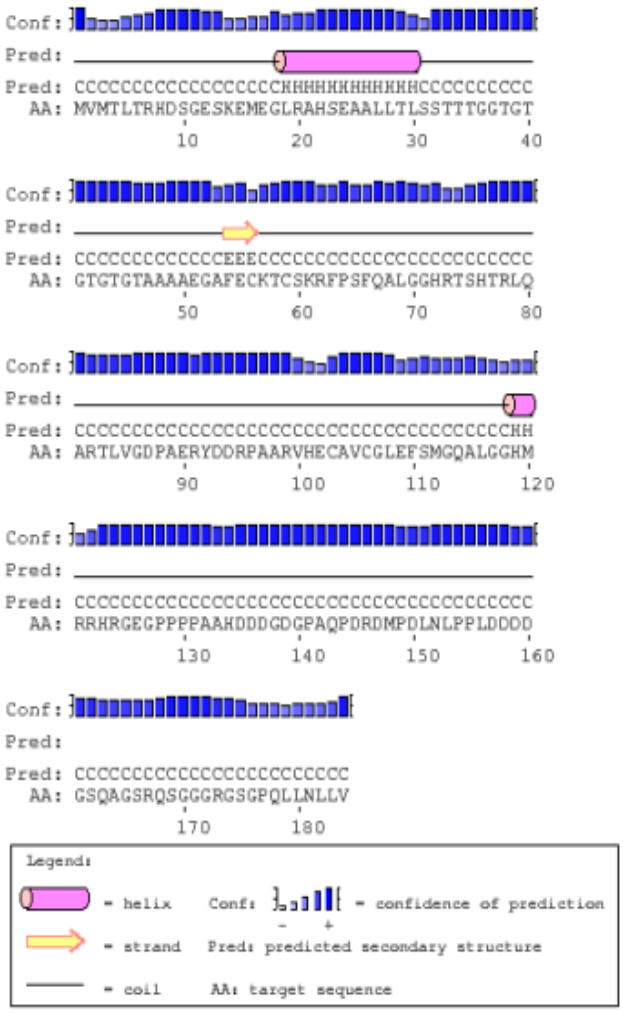


Fig. 2. Predicted secondary structure in amino acid sequence of *ZmZAT8*
The secondary structure was predicted by Psipred. Helix, strand, and coil are mainly part of the secondary structure.

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

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

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 pGBKT7-*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 pGBKT7-*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 constructs were co-transformed into yeast strain Y187, and yeast cells were screened using a synthetic dropout nutrient medium. As shown in Figure 9, yeasts cells harboring the constructs pHis2-*Sh2*/pGADT7-Rec2-*ZmZAT8*, pHis2-Wx/pGADT7-Rec2-*ZmZAT8*or pHis2-*Bt1*/ pGADT7-Rec2-*ZmZAT8*, could grow on SD/−Trp/−Leu 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-Re2-*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*.

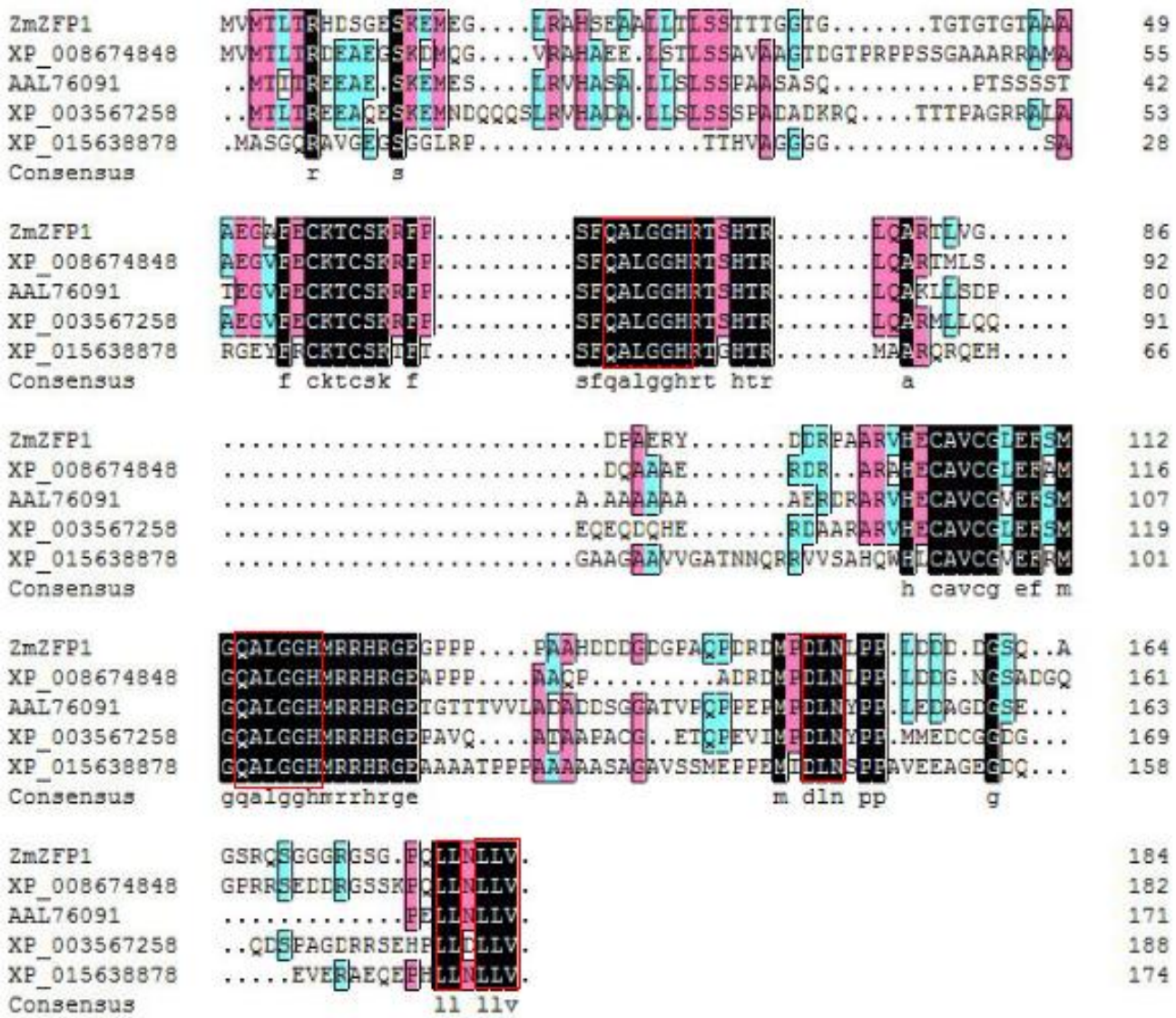


Fig. 3. Multiple alignments of ZmZAT8 proteins

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.

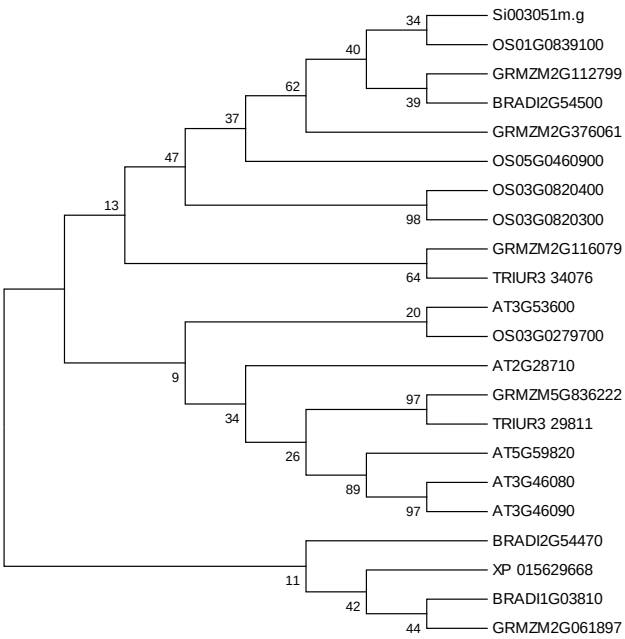


Fig. 4. Phylogenetic tree analysis of *ZmZAT8*
Phylogenetic tree analysis of *ZmZAT8* (GRMZM2G112799) based on amino acids sequences and built using MEGA6.0 software. The tree was generated using the neighbor-joining method with bootstrap support by1000 replicates. The GenBank accession numbers and amino acids equences are listed in supplement 6.

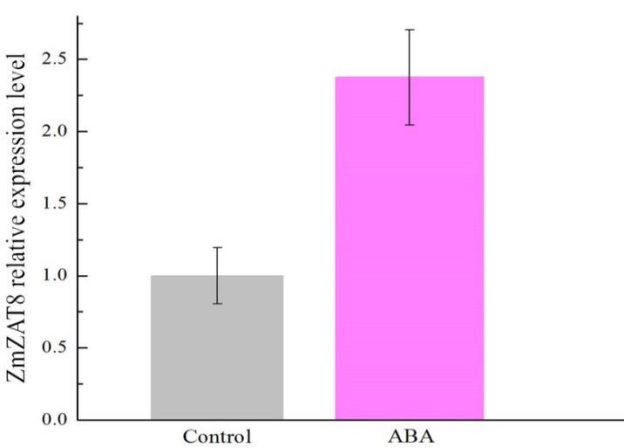


Fig. 5. Real-time qPCR analysis of *ZmZAT8*
Expression of *ZmZAT8* was up regulated by ABA treatment.Ten DAP endosperms were treated with 100 μM ABA for 24 h.

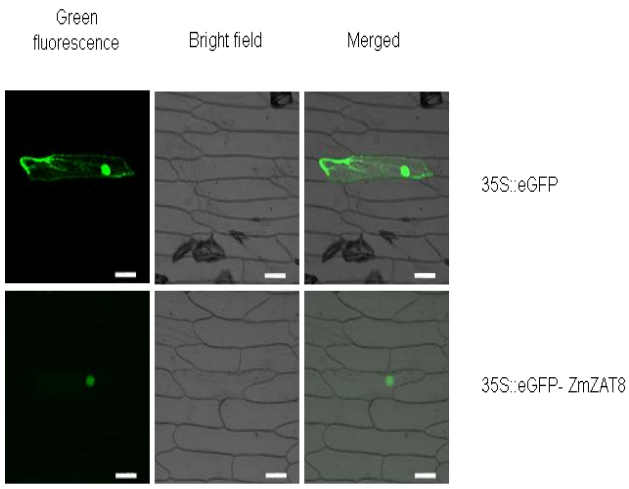


Fig. 6. Subcellular localization analysis of ZmZAT8
Subcellular localization analysis of *ZmZAT8* in onion epidermal cells. Fluorescence microscopy of onion epidermal cells expressing either eGFP, eGFP-*ZmZAT8* as indicated (scale bar=100 um).

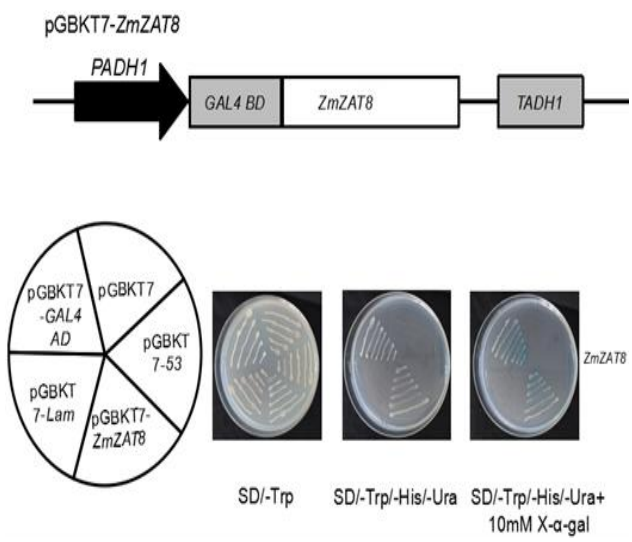
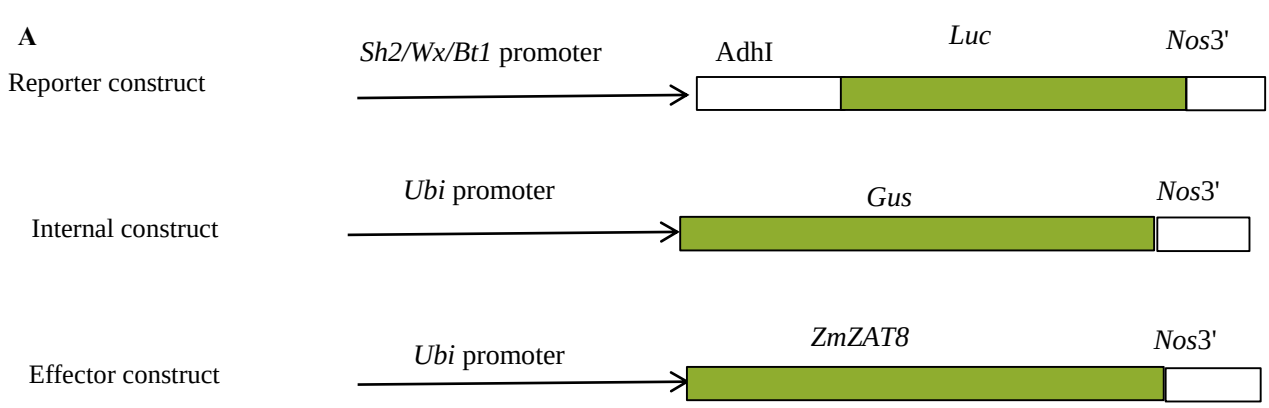


Fig. 7. Transactivation activity assay results for ZmZAT8 in yeast
The structure of the pGBKT7-*ZmZAT8* plasmid; The growth of transformed yeast cells on SD/-Trp, SD/-Trp/-His/-Ura and SD/-Trp/-His/-Ura + 10mg/mL X-α-gal medium, respectively. From the left to the right, the name of map is the corresponding.pGBKT7-GAL4 AD was the positive control. pGBKT7, pGBKT7-Lam, and pGBKT7-53 were negative controls.



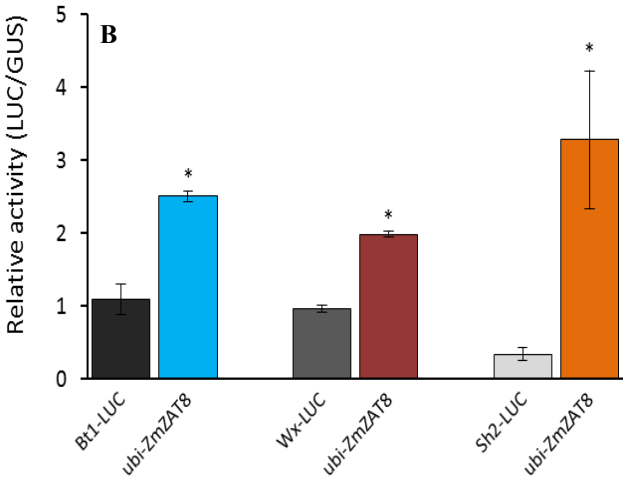


Fig. 8. Transient assay for the interaction between *ZmZAT8* and the promoter of *Sh2*、*Wx* and *Bt1* in maize endosperm.
ZmZAT8 enhances promoter activity of *Bt1*, *Sh2*, *Wx*. (A) Diagram of the reporter construct, internal control construct and reporter construct used in the experiment. (B) Ubi-*ZmZAT8* 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).

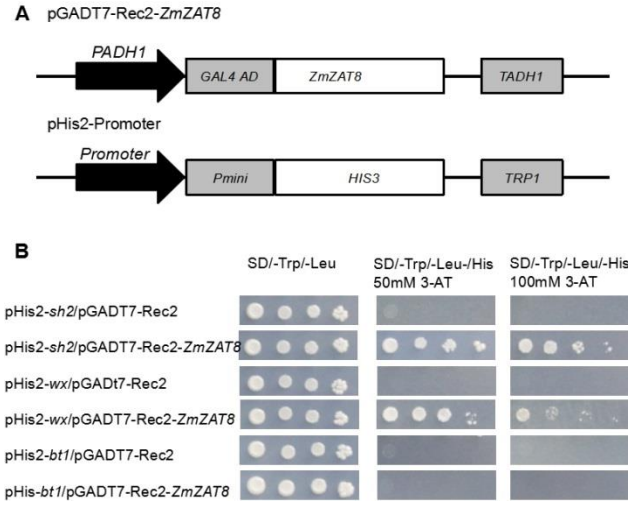


Fig. 9. Identification of *Sh2*、*Wx* and *Bt1* regulated by *ZmZAT8* with yeast one hybrid assay
(A) Schematic structure of yeast expression construct pGADT7-Rec2-*ZmZAT8* and reporter construct pHIS2-Promoter Sh2、Wx and Bt1 promoter; (B) Growth results on defect type medium SD/-Trp/-Leu、SD/-Trp/-Leu/-His 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.

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.

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 (Huai *et al*., 2009; Yu *et al*., 2015). 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). Therefore, it is speculated that ABA induces ZmZAT8 expression to resist stress. Our yeast one-hybrid experiments showed that the expression of *ZmZAT8* alone did not activate the *Bt1* promoter, indicating that the *ZmZAT8* protein did not have a domain that could interact with the *Bt1* gene. However, transient expression experiments demonstrated that *ZmZAT8* could indeed activate *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 AtYY1 is another zinc-finger protein and can directly upregulate the expression of the *ABA 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

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References

Cao, F.Y., K. Yoshioka and D. Desveaux. 2011. The roles of aba in plant–pathogen interactions. *J. Plant Res.*, 124(4): 489-499.

Chen, J., B. Huang, Y. Li, H. Du, Y. Gu and H. Liu. 2011. Synergistic influence of sucrose and abscisic acid on the genes involved in starch synthesis in maize endosperm. *Carbohydr. Res*., 346(13): 1684.

Cutler, S.R., P.L. Rodriguez, R.R. Finkelstein and S.R. Abrams. 2010. Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol.*, 61(1): 651-679.

Doan, D.N P. 1999. The allosterically unregulated isoform of ADP-glucose pyrophosphorylase from barley endosperm is the most likely source of ADP-glucose incorporated into endosperm starch[J]. *Plant Physiol*., 121(3): 965-975.

Fan, L., L. Quan, X. Leng, X. Guo, W. Hu, S. Ruan, H. Ma and M. Zeng. 2008. Molecular evidence for post-domestication selection in the waxy gene of chinese waxy maize. *Mol. Breeding*, 22(3): 329-338.

Hannah, L.C. 2005. Starch synthesis in the maize endosperm. *Maydica*, 50(3): 497-506.

Hannah, L.C., J.R. Shaw, M.J. Giroux and J.Y. Lee. 2001. Maize genes encoding the small subunit of adp-glucose pyrophosphorylase. *Plant Physiol*., 127(1): 173-183.

Hansey, C.N., B. Vaillancourt, R.S. Sekhon, L.N. De, S.M. Kaeppler and C.R. Buell. 2012. Maize (*Zea mays* L.) genome diversity as revealed by rna-sequencing. *Plos One*, 7(3): e33071.

Hennen-Bierwagen, T.A., Q. Lin, F. Grimaud, V. Planchot, P.L. Keeling and M.G. James. 2009. Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: a model for regulation of carbon allocation in maize amyloplasts. *Plant Physiol*., 149(3): 1541-1559.

Hossain, M.A., J.I. Cho, M. Han, C.H. Ahn, J.S. Jeon, G. An and P.D. Park. 2010. The abre-binding bzip transcription factor osabf2 is a positive regulator of abiotic stress and aba signaling in rice. *J. Plant Physiol*., 167(17): 1512.

Hu, Y.F., Y. Li, J. Zhang, H. Liu, Z. Chen and Y. Huang. 2011. Pzss3a, a novel endosperm specific promoter from maize (*Zea mays* L.) induced by aba. *Biotechnol. Lett*., 33(7): 1465-1471.

Hu, Y.F., Y.P. Li, J. Zhang, H. Liu, M. Tian and Y. Huang. 2012. Binding of abi4 to a caccg motif mediates the aba-induced expression of the zmssi gene in maize (*Zea mays* L.) endosperm. *J. Exp. Bot*., 63(16): 5979.

Huai, J., J. Zheng and G. Wang. 2009. Overexpression of a new Cys2/His2 zinc finger protein ZmZF1 from maize confers salt and drought tolerance in transgenic Arabidopsis. *Plant Cell Tiss. Org.,* 99: 117-124.

Huang, H., S. Xie, Q. Xiao, B. Wei, L. Zheng, Y. Wang, Y. Cao and Y. Huang. 2016. Sucrose and aba regulate starch biosynthesis in maize through a novel transcription factor, zmereb156. *Sci. Rep.*, 6: 27590-27590.

Huang, J., X. Yang, M.M. Wang, H.J. Tang, L.Y. Ding, Y. Shen and H.S. Zhang. 2007. A novel rice c2h2-type zinc finger protein lacking dln-box/ear-motif plays a role in salt tolerance. *BBA-Biomembranes*, 1769(4): 220-227.

Jobling, S. 2004. Improving starch for food and industrial applications. *Curr. Opin. Plant Biol*., 7(2): 210-218.

Joseph, M.P. and L. Szabados. 2014. The arabidopsis zinc finger protein3 interferes with abscisic acid and light signaling in seed germination and plant development. *Plant Physiol*., 165(3): 1203-1220.

Klösgen, R.B., A. Gierl and Z. Schwarz-Sommer. 1986. Molecular analysis of the waxy locus of zea mays. *Mol. Gen. Genet*., 203(2): 237-244.

Leff, B., N. Ramankutty and J.A. Foley. 2004. Geographic distribution of major crops across the world. Global Biogeochem. Cy., 18(1): 231-254.

Leung, J. and J. Giraudat. 1998. Abscisic acid signal transduction. *Ann. Rev. Plant Biol*., 49(1): 199.

Li, B. and C.N. Dewey. 2011. Rsem: accurate transcript quantification from rna-seq data with or without a reference genome. *Bmc Bioinformatics*, 12(1): 323-323.

Li, T., X.Y. Wu and H. Li. 2016. A dual-function transcription factor, atyy1, is a novel negative regulator of the arabidopsis aba response network. *Mol. Plant*, 9(5): 650-661.

Mukherjee, S., A. Liu, K.K. Deol, K. Kulichikhin, C. Stasolla and A. Brûlé-Babel. 2015. Transcriptional coordination and abscisic acid mediated regulation of sucrose transport and sucrose-to-starch metabolism related genes during grain filling in wheat (*Triticum aestivum* L.). *Plant Sci*., 240(5): 143.

Patron, N.J., B. Greber, B.F. Fahy, D.A. Laurie, M.L. Parker and K. Denyer. 2004. The lys5 mutations of barley reveal the nature and importance of plastidial adp-glc transporters for starch synthesis in cereal endosperm. *Plant Physiol*., 135(4): 2088-97.

Saito, Y., T. Nakagawa, A. Kakihana, Y. Nakamura, T. Nabika and M. Kasai. 2016. Yeast two-hybrid and one-hybrid screenings identify regulators of hsp70 gene expression. *J. Cell. Biochem*., 117(9): 2109-2117.

Sakamoto, H., T. Araki, T. Meshi and M. Iwabuchi. 2000. Expression of a subset of the arabidopsis cys(2)/his(2)-type zinc-finger protein gene family under water stress. *Gene*, 248(1): 23-32.

Shannon, J.C., F.M. Pien, H. Cao and K.C. Liu. 1998. Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized adp--glucose into amyloplasts of maize endosperms. *Plant Physiol*., 117(4): 1235-1252.

Shure, M., S. Wessler and N. Fedoroff. 1983. Molecular identification and isolation of the waxy locus in maize. *Cell*, 35(1): 225-233.

Smith, A.M., K. Denyer and C. Martin. 1997. The synthesis of the starch granule. *Annu. Rev. Plant Physiol*., 48(48): 67.

Wang, Z., F. Zheng, G. Shen, J. Gao, D.P. Snustad and M. Li. 1995. The amylose content in rice endosperm is related to the post-transcriptional regulation of the waxy gene. *Plant J*., 7(4): 613-622.

Wang, Z., Y. Xu, T. Chen, H. Zhang, J. Yang and J. Zhang. 2015. Abscisic acid and the key enzymes and genes in sucrose-to-starch conversion in rice spikelets in response to soil drying during grain filling. *Planta,* 241: 1091-1107.

Wind, J.J., A. Peviani, B. Snel, J. Hanson and S.C. Smeekens. 2013. Abi4: versatile activator and repressor. *Trends Plant Sci*., 18(3): 125-32.

Yu, L.X., X. Shen and T.L. Setter. 2015. Molecular and functional characterization of two drought-induced zinc finger proteins, zmznf1 and zmznf2 from maize kernels. *Environ. Exp. Bot*., 111: 13-20.

Zeevaart, J. and R. Creelman. 1988. Metabolism and physiology of abscisic acid. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* 39: 439-473.

Zhu, G., N. Ye, J. Yang, X. Peng and J. Zhang. 2011. Regulation of expression of starch synthesis genes by ethylene and aba in relation to the development of rice inferior and superior spikelets. *J. Exp. Bot.,* 62(11): 3907-3916.

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